Annexin 2 and Diabetic Vascular Disease

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Declaration

I Mark Andrew Evans confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Diabetes affects around 2.3 million people in the UK and can lead to both acute and chronic complications. One of the chronic effects of diabetes is the damage that occurs to the vascular endothelium, affecting multiple organ systems, collectively termed diabetic vascular disease. Many proteins have been identified as being altered in diabetes, one of which is annexin 2, a calcium dependent phospholipid binding protein involved in many cellular processes. In response to diabetes annexin 2 is enriched on the surface of the endothelium where it functions as a co-receptor for tissue plasminogen activator and plasminogen to form plasmin, creating a pro-fibrinolytic environment. This thesis aimed to further examine the role of annexin 2 in diabetes, utilising the annexin 2 knockout mouse in both *in vitro* and *in vivo* experiments. As part of our *in vitro* experiments, we identified a hyperglycaemic translocation of annexin 2 to alternative cellular compartments, using sucrose density centrifugation, and an increased susceptibility of VE-cadherin to destabilisation upon the action of vascular endothelial growth factor in annexin 2 knockout endothelial cells. In the *in vivo* experiments we utilised streptozotocin to induce diabetes in the annexin 2 knockout mouse, and examined the progression of both diabetic retinopathy and diabetic nephropathy. Annexin 2 knockout mice developed more severe symptoms of diabetic nephropathy with increased microalbuminuria, mesenchymal matrix expansion and histological changes indicative of renal disease. In contrast to this, symptoms pertaining to diabetic retinopathy were mild in all mice. Non-diabetic annexin 2 knockout mice also exhibited mild hypoglycaemia, potentially implicating defects in the insulin signalling pathway, and suggesting a novel role for annexin 2 in glucose homeostasis.

Contents

List of Figures

List of Tables

List of Acronyms

ACE	Angiotensin converting enzyme
AGEs	Advanced glycation end products
Ang	Angiotensin
AP-1	Activating protein 1
APL	Acute promyelocytic leukaemia
AT1	Angiotensin II receptor – type 1
BBB	Blood brain barrier
BMI	Body mass index
CD	Cluster of differentiation
Cdc	Cell division cycle
CRP	C-reactive protein
CSME	Clinically significant macular oedema
CTGF	Connective tissue growth factor
CTLA	Cytotoxic T lymphocyte antigen
CXCL	Chemokine (CXC motif) ligand
DPX	Di-N-butyle phthalate in xylene
EGF	Epidermal growth factor
EHEC	Enterohemorrhagic Escherichia coli
eNOS	Nitric oxide synthase
ERK	Extracellular signal-related kinase
ET	Endothelins
FAK	Focal adhesion kinase
Flt	fms-like tyrosine kinase
GAPDH	Glyceraldehyde-3 phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GFAP	Glial fibrillary acidic protein
GLUT	Glucose transporter
GM-CSF	Granulocyte macrophage colony-stimulating factor
Grb2	Growth factor receptor-bound protein 2
HIF-1	Hypoxia inducible factor-1
HIV-1	Human immunodeficiency virus
HSP	Heat shock protein
HUVEC	Human umbilical vascular endothelial cells
ICAM-1	Inter-cellular adhesion molecule 1
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IRMA	Intraretinal microvascular abnormalities
MCP-1	Monocytes Chemotactic Protein 1
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein 1 alpha
MMP	Matrix metalloproteinase
NADPH	Nicotinamide adenine dinucleotide phosphate
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NO	Nitric oxide
OCT (procedure)	Optical coherence tomography
OCT (media)	Optimal cutting temperature
PAF	Platelet activating factor
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease-activated receptor
PARP	Poly ADP-ribose polymerase
PI3-Kinase	Phosphoinositide 3-kinases
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PKC	Protein kinase C
PLCγ	Phospholipase-C-γ
PON-1	Paraoxonase 1
Rac1	Ras-related C3 botulinum toxin substrate 1
RAGE	Receptor for advanced glycation end products
Rho	Ras homolog gene family
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
SH2	Src homology 2
SLO	Scanning laser ophthalmoscopy
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T helper
TNF-α	Tumour necrosis factor alpha
t-PA	Tissue-plasminogen activator
UDP-GLcNAc	Uridine diphosphate N-acetylglucosamine
u-PA	Urokinase type plasminogen activator
VCAM	Vascular cell adhesion molecule
VE	Vascular endothelial
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
VRAP	VEGF receptor associated protein
vWF	von Willebrand factor
ZO-1	Zona occludens 1

Chapter 1 Introduction

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1.1 Diabetes

Approximately 2.3 million people in the UK alone are diagnosed with diabetes, with around half a million unaware they have the disease (http://www.diabetes.org.uk/). The disease is characterised by the presence of hyperglycaemia, although it manifests clinically as increased urination, weight loss and lethargy (http://www.diabetes.org.uk). There are three main types of diabetes: Insulin-dependent diabetes (Type 1), Non-insulin dependent diabetes (Type 2) and Gestational diabetes. Recently there have also been reports of a fourth type of diabetes linked to Alzheimer's disease, where it is believed that the progression of the disease leads to a reduction in the ability of the brain to respond to insulin. For this reason, the term 'type-3 diabetes' has been used to describe Alzheimer's disease, with the contribution of insulin signalling to the pathogenesis of Alzheimer's the subject of other investigations[1].

Focusing on type 1 diabetes, unlike the other types it is induced by the actions of the immune system whereby the β-islet cells of the pancreas that produce insulin are lost. This is instigated both by antibodies directed against self antigens in the pancreas (auto-antibodies), and the progressive infiltration of a wide range of destructive auto-reactive immune cells[2]. Much work has been carried out on the progression of type 1 diabetes, which has been shown to require the action of CD4+ (helper, effector) and CD8+ (cytotoxic) T-cells, and macrophages[3,4,5,6,7,8,9,10,11,12,] that usually follows a distinct pathological progression. In the initial stages immune cells accumulate in the pancreas but are not destructive, a triggering event then occurs, the exact cause of which is still unknown, leading to the destruction of the β-islet cells[13,14,15]. Due to the critical role of the immune system in the progression of type-1 diabetes, the immunological response has been well characterised. In brief, since so many immune cells are present in the pancreas at the initiation of destruction the response is often fast and very intense, with T cells, B-cells and macrophages communicating via antigen presentation, cytokines and cell-cell interactions[16]. Macrophages are ultimately responsible for the activation of the other two major cell types, CD8+ cytotoxic T-cells and autoreactive CD4+ effector cells[14], but also contribute directly to the destruction of the β-islet cells by the production of toxic substances such as nitrogen and oxygen derived free radicals[17]. The critical requirement of macrophages for the progression of diabetes has

been demonstrated in a mouse model of diabetes, since if they are removed the generation of the CD8+ T-cell response is inhibited[18,19,20]. The autoreactive CD8+ T-cells destroy the β-islet cells of the pancreas directly through release of toxic substances such as perforin, leading to membrane disruption. However, CD4+ T-cells initiate the activation of B-cells, leading to the production of auto-antibodies that are one of the hallmarks of the disease[14]. CD4+ T-cells also contribute directly to damage of the β-islet cells through the generation of cytokines including IL-1 (Interleukin-1), INF-γ (Interferon gamma) and TNF-α (Tumor necrosis factor alpha) that are secreted into the local environment, leading to membrane disruption, nitric oxide (NO) production and free radicals [15,21,22,23]. Other cytokines such as IL-2 (Interleukin 2), IL-12 (Interleukin 12), IL-17 (Interleukin 17) and IL-18 (Interleukin 18) are also secreted by CD4+ T-cells contributing to the inflammatory state by initiating a T-helper cell type 1 response[22,23]. Even with this vast array of immunological insults, the clinical manifestations of the disease do not occur immediately following activation of the autoimmune response, and usually require approximately 90% of the β-islet cells to have been destroyed[13], hence allowing treatment options to be directed against multiple stages of the disease.

Currently the treatments for type 1 diabetes have three main aims, firstly replacement of the insulin lost as a result of the β-islet cell destruction, protection of the pancreas, and finally regeneration of β-islet function. The most common method of insulin replacement is through subcutaneous injections, which are given to patients with type-1 diabetes on a regular basis. There are also studies ongoing which aim to produce alternative methods of insulin replacement, such as an artificial pancreas[24], however their viability are still under investigation[25]. In terms of restoring β-islet function there are two current approaches, prevention of β-islet cell loss, and regeneration or restoration of β-islet cell mass[26]. Therapies aimed at the prevention of β-islet cell loss are directed at the immunological level either through specific or non-specific immunosuppression whilst therapies aimed at regenerating or restoring β-islet function are focused on transplantation, pharmacological agents and stem cell therapies, however the latter are still in a pre-clinical phase. Apart from the administration of exogenous insulin it is unfortunately the case that no single treatment regimen has shown enough progress to give long-term benefits, and a restorative treatment will potentially have to encompass a number of different therapies to provide any lasting effect.

Type 2 diabetes is the more common form of diabetes[2], and is more prevalent in older people since β-islet function declines over a number of years. It has strong links to obesity and hypertension and is thought to be due to a long term increase in the levels of

insulin in the blood as a result of long term exposure to higher levels of glucose. This then leads to a down-regulation of the insulin receptor and hence ineffective insulin signalling. This 'insulin resistance' then goes on to cause many of the complications found in type-1 diabetes, but often with a less aggressive nature; characterised by a longer duration of the initial symptoms or stages of the condition. There are a number of treatments for type-2 diabetes, many of which are focused on generating more insulin from the pancreas, with control of blood glucose through proper diet and exercise.

Irrespective of the type of diabetes, there are a number of acute and chronic complications that arise. Acute complications include diabetic keto acidoses and non-ketotic hyperosmolar state; the latter more commonly seen in elderly individuals with type 2 diabetes. Chronic conditions of diabetes effect many organ systems and are mostly responsible for the morbidity of the disease. They can be classified into vascular and non-vascular complications, with the latter consisting of skin changes, gastroporesis and sexual dysfunction. The vascular complications can additionally be split into microvascular and macrovascular complications, with peripheral vascular disease, cerebrovascular disease and coronary artery disease examples of macrovascular complications, whilst microvascular diabetic complications comprise diabetic retinopathy, nephropathy and neuropathy.

This thesis focuses on diabetic microvascular complications with particular reference to diabetic retinopathy and diabetic nephropathy. With the diabetic vasculopathies in general, the main force of the hyperglycaemic insult is taken by the endothelial cell, leading to endothelial dysfunction. Since the endothelium is a diverse subject area some of its main functions will be summarised below before describing the dysfunctions that occur as a consequence of diabetes.

1.2 The Endothelium

The endothelium comprises the inner biologically active layer of blood vessels, with direct responsibility for encapsulating the circulating blood. It is also involved in many homeostatic processes. Once believed to be an inert layer of cells, the endothelium in humans has a surface area ranging from 1-7m^2 [27], and is capable of a wide range of responses, with the ability to adapt to widely variable environments. A suitable illustration of this variability is that in the brain, endothelial cells form a tight barrier between one another, using tight junction proteins such as occludin and ZO-1 (zonula occludens-1) to form the blood brain barrier (BBB)[28]. In contrast, endothelial

cells of the liver and spleen take on a discontinuous form to allow the passage of cellular traffic, with the endothelium of the kidney and intestinal villi forming fenestrations, to allow for selective permeability[29]. In accordance with their adaptable nature, endothelial cells in different organs express proteins specific to their function. Signals for endothelial cell differentiation come from the surrounding environment in the form of mechanical forces, paracrine actions of growth factors and interactions with supporting cells such as pericytes and smooth muscle cells. The effect of the microenvironment upon the endothelial cell to define its function has been shown in both *in vitro* and *in vivo* experiments through utilisation of chick/quail transplantation models[30] and culture of endothelial cells on extracellular matrix derived from various organs[31].

Although originally described as a inert layer of cells, the endothelium is not only present to form a structural barrier between the blood and various tissues, but actively participates in maintaining vascular haemodynamics. The main way this is achieved is through the secretion of factors such as NO, prostacyclin (PGI_2), endothelins (ET) and platelet activating factor (PAF). Apart from NO, these factors are not constitutively stored by the endothelium and so their manufacture must be regulated. To briefly summarise their actions; NO is a potent vasodilator[32] and as previously mentioned is constitutively produced, however, the rate at which the endothelium does so can be influenced by various chemical and physical stimuli. NO has many different effects on the vasculature, with one of the most well characterised being it's ability to relax smooth muscle cells[32] leading to changes in blood pressure. A further action of NO is its contribution to wound healing, inhibiting platelet and leucocyte adhesion[33], as well as the binding of fibrinogen[34]. Further to this, NO also has local effects on endothelial cells by enhancing their migration and proliferation[35], whilst inhibiting smooth muscle cell migration[36] and proliferation[37].

In sharp contrast to NO, endothelin-1 (ET-1) has an antagonistic effect on NO production, and is a potent vasoconstrictor[38], with its production stimulated by hypoxia, shear stress and ischemia[39]. ET-1 acts through binding to the endothelin-A (ET-A) receptor present on vascular smooth muscle cells which triggers a rise in intracellular calcium, increasing smooth muscle cell tone[40] and hence blood pressure. The actions of ET-1 on the endothelium can have long lasting effects through the longevity of elevated intracellular calcium, an effect that can be inhibited by the action of NO[41] to restore basal calcium levels. A balance between these two signalling molecules is therefore key to maintenance of vascular tone, and in experimental cases where NO is absent and ET-

1 is allowed to function without antagonism, vasoconstriction and smooth muscle cell proliferation ensue unchecked[42].

Another key action of the endothelium is to maintain the fluidity of the vascular network, such that all the organ systems are constantly perfused. In essence, this is accomplished by inhibiting the adhesion and clotting of platelets on the endothelial cell surface, although there are different ways in which this is achieved. In brief, the control of thrombin generation is crucial, since through its activities as a serine protease it has wide ranging roles in the activation of coagulation factors[43]. Due to this, the endothelium controls thrombin levels by various pathways[44], and very little of the protein is found under healthy circumstances[45]. The extracellular matrix that is secreted by the endothelium, composed of heparan sulphate and other glycosaminoglycans, promotes the activity of thrombin inhibitors such as anti-thrombin III[46], and heparin cofactor II[47]. As well as this mechanism of thrombin control, the endothelium secretes other soluble inhibitors of thrombin, such as thrombomodulin[48] that can bind thrombin, inactivate it, and causes its endocytosis and degradation[49]. In addition to direct binding of thrombin, thrombomodulin can promote anticoagulant pathways such as the actions of anticoagulant protein C[50].

Despite these interventions by the endothelial cell to suppress the generation of thrombi some invariably form, and must be dealt with swiftly if the flow of the vascular network is to be maintained. One of the key proteins involved in this process is plasmin, which is produced from plasminogen through the activities of plasminogen activators. Tissue-plasminogen activator (t-PA) is a commonly found plasminogen activator and when cultured *in vitro* all endothelial cells produce and secrete it into the local environment. However, this is not reflective of the *in vivo* situation as t-PA has been localised to particular subsets of endothelial cells and is more commonly found in the microvasculature, with those negative for t-PA remaining so even upon stimulation to produce it[51,52,53]. Due to its critical role in maintenance of vascular flow t-PA can be activated by various stimuli, but is regulated both at the level of transcription and cellular release to ensure aberrant activity does not occur[54,55]. Another plasminogen activator commonly found is urokinase type plasminogen activator (u-PA), however this derivative is usually found in the context of wound repair and angiogenesis[56]. Although it is not expressed by quiescent endothelial cells[57] u-PA has been shown to be involved in homeostasis since its deletion in mice leads to inflammation-induced thrombi[58].

In situations where the action of plasmin is required at a specific location, endothelial cells can utilise the action of plasminogen receptors that bind both

plasminogen and plasminogen activators, to enhance the formation of plasmin[59,60], with their presence in various contexts reported by several groups[59,61,56,62,60,63]. Other proteins have also been described as plasminogen receptors, one of which is annexin 2[64], which is covered in more detail later.

Although the maintenance of vascular flow and thereby perfusion of the organs of the body is a critical function of the endothelium, in times of injury or damage to the vascular network clots must be formed to limit damage, aid repair and prevent infection. Hence the endothelium is in a dynamic equilibrium between thrombotic and anti-thrombotic states that are balanced by a vast array of extracellular signals[65]. In situations where clotting of the blood, and hence the formation of fibrin is required, a pivotal step is the induction of tissue factor, which occurs rapidly after injury[66], and can be found in atherosclerotic plaques[67,68]. Tissue factor is not normally expressed by quiescent endothelium[69], however, once active it accelerates clotting factor activation, leading to increased mediators of the clotting cascade such as factors X and IX[69]. Tissue factor expression is induced by various stimuli ranging from cytokines to hypoxia[70,71,] and is also enhanced by the products of apoptosis such as anionic phospholipids[72]. When expressed, tissue factor is usually localised to the basolateral surfaces between endothelial cells[73] such that disruption of the endothelial layer is required for it to be mobilised, although some evidence has more recently emerged that it can also be expressed on the surface of endothelial cells[74]. Endothelial cells expressing tissue factor accumulate fibrin on their surface[75], suggesting that they must have binding sites for pro-coagulative proteins, however many of these are as of yet unknown[76]. The best characterised receptor on an endothelial cell for binding of a coagulation protein is protease-activated receptor-1 (PAR-1). PAR-1 is activated by thrombin, whereby an amino terminal fragment of the PAR-1 receptor is released and subsequently binds the remaining portion of the receptor. Once active it leads to the stimulation of both thrombotic and anti-thrombotic pathways, and can disrupt cell-cell contacts[77]. Other PAR receptors PAR-2 and PAR-3, have also been characterised, and in conjunction with PAR-1 they have wide ranging patterns of expression in various tissues of the body[78,79]. Other binding sites involved in coagulation, such as receptors for fibrin and fibrin degradation products, can also be found on endothelial cells[80]. Examples include the 130-kDa protein[81], tissue transglutaminase[82] and $\alpha_V\beta_3$ integrin, although the *in vivo* contexts of these interactions are only now emerging[83,84].

In addition to the maintenance of blood flow, or stagnation in the appropriate context, a more obvious role of the endothelium is the delivery of micro and macro

molecules to the tissues, and regulation of permeability. The endothelium utilises two main routes to transport solutes into the tissue, namely transcellular and paracellular. Transcellular transport utilises intracellular vesicles to move macromolecules from one side of the endothelium to the other through the endothelial cell itself, and is dependent upon proteins such as caveolin[85]. In contrast, the paracellular pathway relies on the regulation of the space between adjacent endothelial cells, allowing solutes and macromolecules to pass between endothelial cells, and is therefore moderated by the proteins that connect endothelial cells to one another, or the endothelial cell junction. Junctions between endothelial cells are comprised of two main compartments: the tight junction, consisting of proteins such as occludin and claudin, and the adherens junctions containing VE-cadherin (vascular endothelial cadherin)[86]. VE-cadherin, as the name suggests, is specific to the endothelium and is a member of the superfamily of calcium dependent adhesive receptors that bind cells together by homotypic interactions, and is crucial for the maintenance of vascular integrity[87]. This is highlighted by the fact that mice deficient in VE-cadherin are embryonically lethal due to aberrant formation of the vascular networks[88,89]. The canomical cadherin structure comprises 5 modular extracellular domains of approximately 110 amino acids each, a transmembrane domain and a conserved cytoplasmic tail[90,91]. The intracellular C-terminal tail is responsible for the interaction between VE-cadherin and the actin cytoskeleton through proteins such as the α, β and γ catenins and p120[92]. The extracellular domain of VE-cadherin has also been investigated and been shown to contain seven-stranded-barrels with structural similarity to the immunoglobulin fold domain[93,94,95,96]. Tight junctions, in contrast to VE-cadherin based adherens junctions, are less common in endothelial cells, but are still important for maintaining the integrity of the endothelium, especially in the blood-brain and blood-retina barriers. A particularly important protein in the tight junction is occludin[97], with various claudins also present, of which claudin-5 appears to be the most abundant[98,99].

As well as mediating transport via the paracellular route, the endothelial junction is crucial in directing and regulating the permeability of the endothelium, and since VE-cadherin is found at all endothelial cell junctions it can be considered key to the maintenance of endothelial paracellular permeability. When the dynamics of VE-cadherin are examined it can be seen that there are a wide range of other proteins are involved in its function. Of particular interest is p120, which functions as a scaffold protein regulating interactions between cadherins, various kinases, phosphatases and

RhoGTPases, which in turn then interact with one another and the catenins to alter the stability of VE-cadherin, and hence endothelial junctions[100]. The importance of p120 in

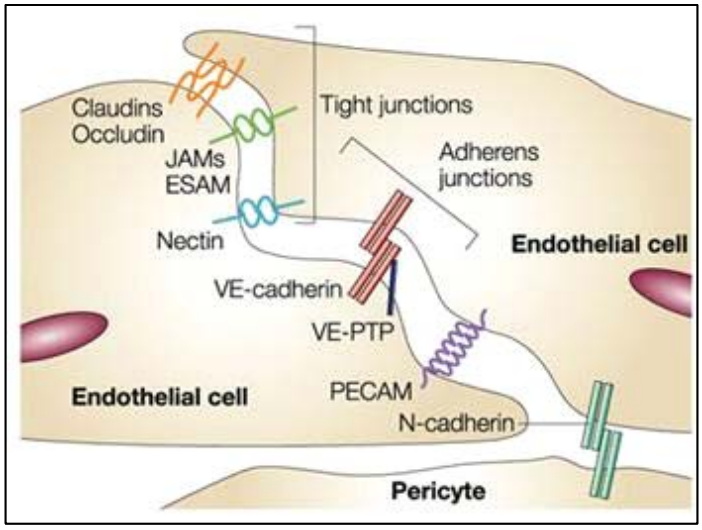

Fig 1.1 Proteins that comprise the junctions in endothelial cells.
The junctions between endothelial cells consist of various protein interactions with tight, adherens and pericyte interactions displayed. Image adapted from Dejana, E. (2004). Endothelial cell junctions: happy together. *Nat Rev Mol Cell Biol.* Apr;5(4): p261-270

VE-cadherin stabilisation is further highlighted by reports that an increase or decrease in p120 leads to a greater or lesser amount of VE-cadherin found at the surface of the endothelial cell[101,102] with the interplay between proteins subsequently recruited by p120 also important in the stabilisation of endothelial adherens junctions[103]. Various proteins can destabilise VE-cadherin, and hence increase the permeability of the endothelium. For example thrombin reduces VE-cadherin surface content, and causes phosphorylation of the protein through the actions of PKC-α (Protein kinase C alpha), to a form that is less stable than its native form[103]. In addition to phosphorylation, destabilisation can also be mediated by agonists such as VEGF, which induces internalisation of the protein[104]. In sharp contrast, stabilisation of VE-cadherin is centred around p120, especially since the internalisation of VE-cadherin gives rise to an increase in p120, subsequently reducing destabilising proteins such as RhoA (Ras homolog gene family, member A), whilst increasing Rac1 (Ras-related C3 botulinum toxin substrate 1) and cdc42 (cell division cycle 42) that function to stabilise VE-cadherin[105]. These stabilisation events are then further supported by the de-phosphorylation of VE-cadherin and the subsequent re-association of p120[106].

With the wide diversity of roles, and multiple actions required to maintain stability, the endothelium is very biologically active with a high rate of protein synthesis. Endothelial cells utilise both fatty acids and glucose as energy sources, and

whilst most tissues of the body selectively transport glucose in response to demand using the insulin regulated GLUT-4 transporter, the main transporter of glucose in endothelial cells is GLUT-1. GLUT-1 is functionally different to GLUT-4 in that it is a constitutively open channel for glucose once inserted into the membrane, essentially ensuring that the glucose concentration of the cytosol is equivalent to that of the blood. Regardless of this, the majority of the glucose that reaches the endothelium is not utilised by the cell itself but delivered to the underlying tissue. Endothelial cells achieve this in two ways as outlined above, either by permitting glucose flow between cell-cell junctions, or by transcellular transport. In the brain the blood brain barrier (BBB) created by the endothelium, is sufficiently tight that the brain must rely on transcellular transport alone to ensure the brain is supplied with glucose. This is achieved through distribution of GLUT-1 transporters on the basal surface of the endothelium, however the true contribution of the GLUT-1 receptor to glucose homeostasis of the underlying tissues has not been fully investigated.

1.3 Endothelial dysfunction

The correctly functioning endothelium is adaptable to many different physiological conditions and stimuli, however in diabetes the endothelium becomes compromised by hyperglycaemia. This process is termed “Endothelial dysfunction” and is characterised when the response of the endothelium is inadequate to the demands requested of it. As mentioned previously, endothelial intracellular glucose is equilibrated to the blood glucose concentration through the actions of the GLUT-1 transporter. Therefore, the increase in intracellular glucose concentration in endothelial cells, along with the plethora of growth factors and other vasoactive compounds influenced by diabetes, produces the endothelial dysfunction seen in diabetic microvascular and macrovascular complications.

The molecular mechanisms of hyperglycaemia in endothelial cells comprise four main pathological processes[107]. These are increased flux of glucose through the polyol pathway, an increase in advanced glycation end products (AGEs), increased activation of protein kinase C isoforms and increased hexosamine pathway activity.

Considering first the polyol pathway, when intracellular glucose becomes too high one of the enzymes affected is aldose reductase. This enzyme is usually responsible for removing toxic aldehydes from the cell, by reducing them into inactive alcohols. This process requires the co-factor NADPH for the reduction reaction, but

when intracellular glucose concentrations become too high aldose reductase instead uses NADPH to reduce glucose to sorbitol. The consequence of this is the reduced availability of NADPH, which is involved in many different biological processes. One such process important in the progression of diabetes, is the requirement of NADPH for the regeneration of reduced glutathione; an anti-oxidant. Hence with reduced levels of NADPH, the cell is more vulnerable to toxic free radicals that can accumulate in hyperglycaemic conditions. *In vivo* evidence for this was shown in a study by Engerman *et al* (1994), where pharmacological inhibition of aldose reductase in diabetic dogs improved the neurological conduction velocity in comparison to their control cohorts[108].

Advanced glycation end products are formed either through the Maillard reaction (non-enzymatic glycation) or via other compounds such as methylglyoxal. In the presence of hyperglycaemia both the availability of glucose for the Maillard reaction and the concentration of methylglyoxal are increased, leading to a concurrent increase in AGEs. These products can have three main effects, if formed in the cytosol they can go on to damage intracellular proteins, disrupting their function[109]. Secondly, if formed or secreted into the extracellular environment they can go on to activate the AGE receptor[110], or thirdly crosslink extracellular proteins such as those that form the basement membrane of the endothelium, causing it to thicken or harden[111].

As well as interfering with enzyme action and the formation of AGEs, hyperglycaemia increases the amount of diacylglycerol in the cell. Diacylglycerol is the critical activation co-factor for many protein kinase C isoforms and its increase leads to an up-regulation of the activity of these proteins, and their various downstream signalling pathways. Not all these pathways have been implicated in the progression of diabetes, but some of those that have are outlined here. eNOS and endothelin-1 are two proteins, negatively and positively regulated respectively by PKC, that are involved in management of vascular tone, and hence blood flow. In hyperglycaemic conditions the activation of these proteins by PKC is increased, leading to vasoconstriction. Increased PKC can also up regulate the activity of other proteins such as VEGF (vascular endothelial growth factor), TGF-β (Transforming growth factor beta), NF-κB and NADPH oxidases, leading to other complications such as vessel occlusion, leucocyte attraction, oedema and increased reactive oxygen species (ROS).

The fourth and final process that has been shown to be affected by hyperglycaemia is the hexosamine pathway. This pathway describes the initial metabolism of glucose, where it is converted first to glucose-6-phosphate and then fructose-6-phosphate. Under normal circumstances the fructose-6-phosphate would then

be used in the glycolytic pathway to produce ATP. However, when hyperglycaemia is present the concentrations of these metabolites increase and some of the fructose-6-phosphate gets diverted via the enzyme glucosamine:fructose-6-phosphate amidotransferase to produce glucosamine-6-phosphate, and then finally uridine diphosphate N-acetylglucosamine (UDP-GLcNAc). This compound then targets serine or threonine residues of transcription factors leading to changes in their activity and subsequently their genetic targets, causing disruption throughout the cell.

A recent publication proposed a linking mechanism for these four pathways whereby hyperglycaemia leads to an increase in superoxide production potentiating the activity of the enzyme poly ADP-ribose polymerase (PARP)[107]. This in turn down-regulates a key glycolytic enzyme glyceraldehyde-3 phosphate dehydrogenase (GAPDH), leading to an increase in glyceraldehyde-3 phosphate and other metabolites further upstream. This then creates substrates for the four previously mentioned pathways since glyceraldehyde-3 phosphate is one of the major compounds responsible for the formation of AGEs, and also is used to produce diacylglycerol, hence increasing the activity of PKC. Further upstream increased fructose-6-phosphate participates in the hexosamine pathway and abberant glucose metabolism in the polyol pathway.

In addition to the direct actions of hyperglycaemia on the endothelial cell, hyperglycaemia can also induce changes in the activity or abundance of molecules in the local environment that can affect endothelial cell function. TGF-β, TNF-α, CRP (C reactive protein) and VEGF all fall into this category and shall be explored briefly here. The actions of TGF-β are well defined in a number of vascular pathologies [112], however in diabetes it becomes activated by the actions of hyperglycaemia induced PKC activation, AGEs and other cytokine derived activation of endothelial cells[113]. Upon contact with endothelial cells TGF-β has a number of functions ranging from changes in the rate of cell growth, to cellular differentiation and apoptosis. TGF-β is also a potent inducer of the proteins that make up the basement membrane of endothelial cells such as collagens I and IV, fibronectin, laminin and proteoglycans, and as such is thought to play a significant role in fibrosis phenotypes, seen in conditions such as diabetic nephropathy[114].

TNF-α is a pro-inflammatory cytokine produced by many cell types which can have various effects on the endothelium. These include an increase in vascular permeability, altered vasoregulatory responses, activation of NF-κB and an increase in coagulation through inhibition of anti-coagulant proteins and activation of pro-coagulant activity[115]. Induction of NF-κB activity by TNF-α has various effects on

endothelial cells including the up regulation of ICAM-1 and other cellular adhesion factors such as VCAM and E-selectin that lead to the recruitment of immune cells, and subsequent inflammation. NF-κB also induces the expression and activity of pro-inflammatory cytokines such as IL-1, IL-6 and IL-8, leading to further non-specific inflammation that is characteristic of some diabetic microvasculopathies such as diabetic retinopathy.

In addition to the actions of NF-κB mediated through TNF-α, levels of CRP, an important pro-inflammatory, pro-atherogenic protein, are increased in both type-1 and type-2 diabetes[116,117]. Its effects on endothelial cells include decreased NO and prostacyclin production, an increase in cell adhesion molecules and increase in ET-1, MCP-1 (monocyte chemotactic protein-1), IL-8 and PAI-1 (Plasminogen activator inhibitor-1) production, leading to changes in vascular tone, inflammation and fibrin production. It can also exert its effects on endothelial cells by acting on the supporting smooth muscle cells of the vasculature, further increasing NO and NF-κB as well as increasing angiotensin II (Ang II) signalling, resulting in the increased production of reactive oxygen species (ROS). Lastly VEGF signalling in the context of diabetes in the endothelial cell is of particular importance, and is thought to play a significant role in many diabetic vasculopathies. As such the contribution of VEGF to diabetic vasculopathies will be discussed in detail later.

Once affected by the actions of diabetes, the endothelium is said to become dysfunctional, however, the term “Endothelial dysfunction” is not indicative of a single condition, but instead describes the situation where one or more of the constituent functions of the endothelial cell are altered to the detriment of the overall functioning of the vasculature. One of the more classical types of endothelial dysfunction in the context of diabetes is thickening of the basement membrane, through the actions of TGF-β and other contributory factors[118,119]. In this situation dermatan sulphate proteoglycans and chondroitin sulphate proteoglycans are increased, whilst heparan sulphate proteoglycans are drastically reduced[120]. Since the extracellular matrix requires a large quantity of heparan sulphate proteoglycans for correct functioning, a reduction in these leads to dysfunction of the endothelium evident in reduced NO availability, increased adhesion of mononuclear cells and platelets, and leakage of macromolecules across the endothelial layer. These in turn lead to changes in vascular tone, inflammation and aberrant fibrin formation.

Microalbuminurea is also a common consequence of endothelial dysfunction, however it’s exact mechanisms are not clearly defined. Changes in hydrostatic pressure,

the selectivity of the glomeruli, disruption of the supporting cells (podocytes) in the glomeruli interactions with the endothelium and decreased protein reabsorption in the kidney are all thought to contribute; concepts that will be explored in more detail later upon examination of diabetic nephropathy since microalbuminuria is a defined constituent of the disease progression[121,122].

As mentioned previously, a major function of the endothelium is to maintain the fluidity of the blood and to prevent clot formation unless required to promote wound healing or protection from infection. The presence of diabetes can influence the sensitive dynamics in this process leading to an increase in thrombus formation. Such events are mediated by an increase in soluble thrombomodulin and inhibitors of t-PA, with a concurrent decrease in tPA itself, leading to an inability to promote anticoagulant pathways, and hence create and maintain fibinolytic environments [123,124]. Increased von Willebrand factor (vWF) levels have also been correlated with increased risk of cardiovascular complications in diabetes patients[125,124], and is of particular interest regarding thrombus formation since it is involved in the adhesion of platelets and complexes with clotting factor VIII.

Endothelial repair is usually carried out via angiogenesis, but in the hyperglycaemic environment this is yet another process that becomes dysfunctional. In the case of wound healing the angiogenesis response is inadequate resulting in reduced wound healing, aberrant responses to vascular occlusion and an increased risk of myocardial infarction[126]. However, the opposite can also be seen in other diabetic pathologies such as diabetic retinopathy where angiogenesis is allowed to progress unchecked, although in both conditions it is a reduced blood supply that stimulates the response.

Through the actions of numerous processes diabetic endothelial dysfunction can give rise to multiple organ system complications particularly affecting the kidney, heart and eye. In these organs it is the microvascular networks that bear most of the insult, two such examples of diabetic microvasculopathy commonly found in diabetic patients being diabetic retinopathy and diabetic nephropathy.

1.4 Diabetic retinopathy

In the eye the damage to the vascular network of the retina is characterised by a condition called diabetic retinopathy. Diabetic retinopathy is the leading cause of legal

blindness in the working population in the US, and a significant proportion of diabetics will present some signs of the disease within 15 years of diagnosis.

The eye consists of multiple layers of cells, which collectively ensure light is focused onto the light-sensing part of the eye, namely the retina. In essence the retina is comprised of four major cell types, neurons (photoreceptors, ganglion, bipolar, horizontal and amacrine cells), microglia (resident macrophages) macroglia (Müller cells and astrocytes), and finally the microvascular cells, comprising the endothelium and pericytes. In brief, the neurons detect light both in intensity and spectrum and deliver that information to the optic nerve which is subsequently directed to the visual cortex of the brain. The glial cells (macro- and micro-) are positioned between the blood vessels and neurons such that they can provide nutritional and regulatory support and span the entirety of the retina, detecting and responding to any immunological cue that may arise, and clearing necrotic and dead cells. This leaves the retinal vasculature, consisting of the primary, inner deeper and outer deeper plexuses. The former of these originates from the optic nerve head in development giving rise to the deeper plexuses later in development. During vasculogenesis of the retina, supporting cells such as pericytes are recruited which strengthen the barrier between the blood and retina. The ratio of pericytes to endothelial cells can often approach values of 1:1, highlighting the importance of vascular integrity, a subject that will be commented upon later. Further information regarding the development and constituents of the retinal vasculature can be found in a recently published review[127].

A large part of the detectable changes associated with the progression of diabetic retinopathy involve the vasculature, but the other cells that make up the retina are also affected by hyperglycaemia. Macroglia, for example, release inflammatory mediators and change their levels of certain proteins. In astrocytes this is seen in the down regulation of an intermediate filament protein GFAP (Glial fibrillary acidic protein)[128,129]. Since the function of the astrocyte is closely linked to it's levels of GFAP, down-regulation of this protein is indicative of reduced astrocytic function. Conversely, in Müller cells GFAP is up-regulated in response to hyperglycaemia[130], even before the clinical signs of diabetic retinopathy are evident, in addition to expressing less glutamine synthetase[131,132]. Microglia also become activated through the actions of hyperglycaemia[129,133], undergoing proliferation and becoming more active, expressing proteins such as OX-42. Both types of glial cell can also express VEGF (vascular endothelial growth factor), a cytokine that shall be commented upon in detail later.

One of the initial symptoms of diabetic retinopathy is the presence of microaneurysms, usually located in the inner nuclear layer of the retina. These are a hallmark of diabetic retinopathy[134,135,136], and usually appear as dark red or white spots upon examination of the eye using ophthalmoscopy. The steps required to form a microaneurysm have yet to be fully described but are thought to involve damage to the basement membrane[137] and require changes in endothelial proliferation[138], vascular flow[139,140], and increased pericyte cell death[141,142]. Indeed, pericyte cell death appears to

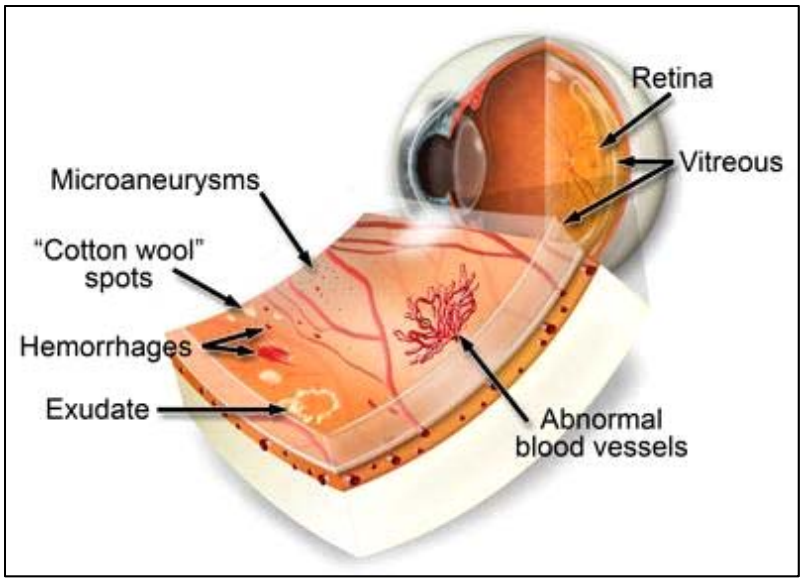

Fig 1.2 Manifestations of diabetic retinopathy.

Various abnormalities can be detected at the back of the retina that indicate progression of diabetic retinopathy, ranging from microaneurisms through to haemorrhages. Image adapted from http://emeraldeye.com/images/DiabeticRetinopathyLabeled.jpg

be critical for the formation of microaneurysms, since several studies have shown that microaneurysms consistently lack pericytes upon histological analysis[143,144]. In addition to this, 'pericyte ghosts', or the residual basement membrane left by an apoptosed pericyte, can often been seen close to the site of a microaneurysm, further suggesting that pericyte death contributes to their formation[136,145,146]. The apoptosis of these supporting cells is, however, not the only factor in the formation of microaneurysms, and localised increases in capillary lumen pressure are also thought to play a role[147]. This concept is further supported by the observations of microaneurysms in microvascular diseases that are not characterised by the loss of pericytes, such as hypertension, branch retinal vein occlusion, Coats' disease and sickle cell anaemia. Once formed, various cell types have been reported to populate microaneurysms, which change as it matures. In early microaneurysms immune cells and resident endothelial cells can be found, whilst in later stages the endothelium dies leaving only erythrocytes visible by histology.

The loss of pericytes is hard to detect at a diagnostic level, however, animal models have given us insight into the mechanisms of pericyte loss. The vasculature is particularly resistant to enzymatic digestion, whereas the remainder of the cells that make up the retina are not. For this reason the retina can be treated with high levels of trypsin to digest away the neurons and other supporting cells, leaving the vasculature behind. When this is done the loss of pericytes can be detected as pockets in basement membranes that do not colour with nuclear stains and which are then classified as 'pericyte ghosts'[136,142]. The microaneurysms themselves can also be visualised using this technique, and can be seen as discrete regions where the vasculature has 'ballooned out' and the blood is allowed to pool.

The exact cause of pericyte loss in diabetic retinopathy is unclear. Previous studies have noted that pericytes have a particularly high expression of the enzyme aldose reductase, which converts glucose to sorbitol and, in the presence of hyperglycaemia, can cause the accumulation of sugar alcohols[148]. It was also suggested by these authors that this would be a feasible mechanism for specific pericyte loss since other retinal cells, such as endothelial cells, did not express aldose reductase at such high levels[149]. However, the opposite has now also been shown in other studies, suggesting that the actions of aldose reductase in hyperglycaemia may not be the only mechanism of pericyte loss. The accumulation of AGEs in pericytes is another proposed mechanism for pericyte loss through the actions of the receptor for advanced glycation end products (RAGE)[150]. In spite of this, the timing of AGE accumulation is inconsistent with the timing of pericyte loss[151], suggesting that this also is not the only mechanism. In essence, pericyte loss in diabetic retinopathy is more likely to be due to the contribution of multiple factors including aldose reductase and AGEs and also cytokines such as TNF-α[152,153] and the actions of NF-κB[154].

Other physical manifestations of diabetic retinopathy that usually present after pericyte loss include venous beading, looping or segmentation. Haemorrhages can also be found, often in the middle retinal layers, and are classified as dot or blot haemorrhages. As mentioned previously, capillaries can become occluded either by the action of thrombi, or static leukocytes. When this occurs ophthalmologists can often detect what are described as cotton wool spots, resulting from occlusion of capillaries in the nerve fibre layer. Intraretinal microvascular abnormalities (IRMA), are then often detected close to areas of capillary occlusion, and are observed as dilated capillaries functioning as collateral channels for the flow of blood.

As mentioned previously, ophthalmologists use the physical manifestations of diabetic retinopathy to screen for the disease, hence a scale of severity of the condition has been created to give some indication of its progression. There are four classifications of non-proliferative diabetic retinopathy: mild, moderate, severe and very severe[155]. The presence of one or more microaneurysms without any further symptoms is sufficient to classify the disease as mild[156], and an increase in number, in addition to hemorrhages, cotton wool spots, venous beading and IRMA within set frequencies and distributions then mark the disease as 'moderate'. Further progression to the 'severe' and 'very severe' categories are met by the increase in frequency of these symptoms and their increasing distribution throughout the retina, in accordance with standard photographs[155] and measurements of the retina.

In terms of the visual changes associated with these stages of diabetic retinopathy, it is not the previous changes that cause the majority of symptoms, but the associated macular oedema. This occurs when fluids and exudates from the blood vessels affected by the hyperglycaemia leak into the macula, between the outer plexiform layer and inner nuclear layer, causing it to swell, distorting vision and making it appear 'out of focus' through the loss of 'fine' vision. Macular oedema is thought to occur through either breakdown of the junctions between endothelial cells and/or the loss of pericytes[157,158,159], or alterations in paracellular transport. Upon initial examination of the eye ophthalmologists often use the formation of hard exudates (lipoprotein deposits between the inner plexiform and inner nuclear layers of the retina), to indicate signs of macular oedema, although it can also be detected by the use of optical coherence tomography (OCT). Towards the later stages of the disease the fluid can spread to all the layers of the retina, exacerbating the alterations in vision. Clinically, macular oedema is classified as retinal thickening within 1 disk diameter from the centre of the macula[160,161], but can progress to 'clinically significant macular oedema' (CSME) if one of the following criteria are satisfied: "retinal thickening or hard exudates within 500μm of the centre of the macula and/or zone(s) of retinal thickening 1 disk area in size at least in part of which is within 1 disk diameter of the macula centre"[160]. In addition to the CSME classification, macular oedema can be characterised as focal or diffuse. Focal macular oedema is usually the result of leakage from microaneurysms, or discrete areas of the vasculature[162], usually surrounded by hard exudates, whereas diffuse macular oedema is indicative of generalised leakage from the capillaries, IRMAs or in severe cases the macrovasculature[163,164]. Although spontaneous recovery from macular oedema is not uncommon, treatment is important

since in approximately a quarter of cases the absence of treatment will result in moderate vision loss within three years [165,166].

Vascular leakage can be detected by a method called fluorescein angiography. This utilises a fluorescent dye which is injected intravenously in humans, or into the peritoneal cavity in animal models of diabetes, and subsequently detected in the retinal vasculature by scanning laser ophthalmoscopy (SLO) as it perfuses systemically. If the retinal vasculature is intact the dye will remain in the vessels and will be safely excreted, however, if the vasculature of the retina is damaged and vascular leakage is occurring the fluorescent dye will leak out into the surrounding tissue, detectable as a widening area of florescence at the point of leakage. It is important to note, however, that the presence of vascular leakage is not always indicative of retinal swelling, since leakage is required to exceed the capacity for removal by the retinal pigment epithelium (RPE) pump before swelling will occur.

Up until this point all the described effects fall into the category of 'non-proliferative diabetic retinopathy', the disease then has two further characterisation points that are set as early or high-risk proliferative diabetic retinopathy, the former defined as the presence of newly formed blood vessels and the latter as the haemorrhage of those vessels, where the patient is then at a high risk of irreversible vision loss either through obscured vision or retinal detachment[167]. Although there are many cellular changes attributable to the pathogenesis of diabetic retinopathy and these are accountable to hyperglycaemia and other factors, one signalling molecule seems consistently involved with the majority of the changes, namely vascular endothelial growth factor (VEGF).

VEGF is a dimeric glycoprotein of approximately 40kDa that, as suggested by it's name, stimulates endothelial growth through enhancement of proliferation, migration and tube formation, leading to the formation of new vessels. It consists of seven family members with VEGF-A commonly referred to as 'VEGF', and VEGF-B to VEGF-F with placental growth factor (PlGF) constituting the six remaining members. In addition to these family variations, the VEGF gene can be alternatively spliced producing proteins of different solubility, linked to their ability to bind heparin, with $VEGF_{206}$ and $VEGF_{189}$ binding strongly to heparin, and hence the extracellular matrix, keeping them effectively insoluble. $VEGF_{121}$ however, is unable to bind heparin and is hence freely soluble, with $VEGF_{165}$ moderately able to bind heparin and hence moderately soluble. As well as these angiogenic forms of VEGF there have recently been discovered dominant negative forms of VEGF, called $VEGF_{xxxb}$, that whilst

largely homologous have subtle changes at their C-terminal ends that only partly activate their receptor[115] leading to a dominant negative type effect. The functional significance of these dominant negative forms of VEGF has also recently come to light with evidence suggesting they are down-regulated in both physiological angiogenesis[168] and pathological angiogenesis[169], highlighting their central role in vascular homeostasis.

The actions of VEGF are mediated through VEGF receptors, of which there are 4 members: VEGFR1 (VEGF receptor 1), VEGFR2 (VEGF receptor 2), Flt-3 (fms-like tyrosine kinase-3) and Flt-4 (fms-like tyrosine kinase-4). Each has its own specificity for the different VEGF family members, but broadly speaking VEGFR1 and VEGFR2 are involved in angiogenesis[170], whereas Flt-3 and Flt-4 are involved in haematopoiesis and lymphangiogenesis[171]. Regardless of this, they all have a 750aa extracellular domain constituting seven immunoglobulin like domains, a single transmembrane domain, followed by a split tyrosine kinase domain and a C-terminal tail. Activation of VEGF receptors requires their dimerisation and they form either hetero- and homo-dimmers to achieve this[172]. Once dimerised and activated, auto-phosphorylation of key residues occurs on the intracellular tail, which leads to the binding and activation of SH2 domain containing proteins, and subsequent signalling cascades. VEGFs also bind to a further type of receptor called the neuropilins that function as co-receptors for VEGF. The neuropilins have various functions with neuropilin-1 contributing to endothelial migration and adhesion, whilst other forms contribute to bone-marrow derived cell differentiation into vascular precursors[173] and synthesis and release of prostacyclin[174]. Due to the complexities of the different isoforms and splice variants of VEGF proteins, with the additional complexities of the VEGF receptors, the downstream signalling pathways of activation are wide ranging. Focusing on VEGFR2, one of the main receptors activated by VEGF, surprisingly few proteins have been shown to directly interact with the receptor. These that do include phospholipase-C-γ (PLCγ), Shb, Sck, VRAP (VEGF receptor associated protein) and annexin 5[175]. Briefly, activation of PLCγ in the context of VEGF signalling activates MAP Kinase signalling, stimulating proliferation[176], and also activates protein kinase-C via DAG and elevation of intracellular calcium. Shb, however, stimulates endothelial cells to migrate and can activate PI3 Kinase that subsequently influences actin dynamics through Rac and p38 MAP kinase signalling, or serves to activate Akt and enhance cell survival[177], in addition to increasing NO production through eNOS activation. Sck binding to the VEGFR2 results in its association with Grb2 that in turn activates MAP kinase, with VRAP activation by VEGFR2 binding important in migration, and regulation of Src

signalling through direct interactions. There are also pathways downstream of VEGF receptor activation that have not been completely elucidated, such as signalling through FAK (focal adhesion kinase) to paxillin that regulates focal adhesions.

In the context of diabetic retinopathy it is often the lack of oxygen (hypoxia) that induces VEGF levels to rise. Hypoxia upregulates VEGF levels by many different mechanisms[178] including increased transcription, increased stability of mRNA, differential protein translation using IRES sequences, and increased chaperone protein expression. Increased transcription of VEGF in hypoxic conditions is mostly attributable to HIF1 (hypoxia inducible factor-1), a heterodimer consisting of HIF1-α and HIF1-β. The latter, HIF1-β is constitutively expressed whilst levels of the former, HIF1-α, are responsive to oxygen levels in the cell[179]. Under normoxia HIF1-α is targeted for degradation, however under hypoxia these mechanisms are inhibited and HIF1-α levels increase[180], HIF1-α then binds HIF1-β where it is translocated to the nucleus and binds the hypoxia responsive element of the VEGF gene, initiating transcription[181,182,183,184,185].

The second method through which VEGF levels are up regulated is mRNA stabilisation. The mRNA of VEGF is unusually unstable in comparison to that of most other eukaryotic genes, with a half-life of only 1-2 hours instead of the more normal 10-12 hours. However, hypoxia has been shown to increase the half-life of VEGF mRNA 2-3 fold[186], due to the stabilising effect of HuR, a 36kDa RNA binding protein, which binds to the 3'-UTR of VEGF mRNA preventing its degradation[187,188]. Thirdly, IRES sequences, specific sites of attachment for the ribosomal machinery that override the normal methods of translation have been found in the 5'-UTR of VEGF mRNA[189]. Lastly, up-regulation of a chaperone protein, ORP150 that shuttles VEGF from the endoplasmic reticulum to the Golgi apparatus has been shown in the context of hypoxia to facilitate VEGF transport and secretion[190].

VEGF is not only regulated by oxygen levels, it has also been shown to be regulated by other proteins such as IGF-1. IGF-1 is critical for vessel development *in vitro*[191,192], with pre-term infants with reduced serum levels of IGF-1 showing a higher incidence of retinopathy[193]. Despite this, IGF-1 does not directly influence the levels of VEGF protein, as demonstrated in mouse knockout models[194], but does so by decreasing Akt activation in the context of VEGF signalling, which has been shown to be important for endothelial cell survival[195].

In terms of the effects of VEGF on diabetic retinopathy, it has been described as the primary mediator of vascular alterations in disease progression[196,197,198]. Indeed

many of the changes associated with diabetes such as hyperglycaemia and production of AGE have been noted to increase VEGF expression, and the levels of VEGFR2[199,200]. There is also a strong correlation between VEGF levels in the eye and development of proliferative diabetic retinopathy[196], with therapies designed to block the actions of VEGF shown to decrease the associated permeability defects of diabetic retinopathy both in patients[201,202,203,204] and animal models[205,206,207].

Many factors thus contribute to the increased expression of VEGF including hypoxia, growth factors, inflammatory cytokines and ROS. Each of these has been shown to up-regulate VEGF in disease models but not all have yet been characterised in the context of diabetic retinopathy. Hypoxia is by far the most studied regulator of VEGF expression in diabetic retinopathy, with increases in capillary density linked to hypoxic regions of the retina[208]. However, up-regulation of VEGF occurs as early as 1 week post diabetes, when hypoxic regions of the retina would not have yet formed, suggesting that other factors may play a role its up-regulation. Such examples include TGF-β, TNF-α, IGF-1 (discussed earlier), AGEs and oxidative stress[209,210,211,212,213]. Whilst their mechanisms of action are unclear, all have been shown to up-regulate VEGF in the context of diabetic retinopathy.

ROS, also up-regulated in diabetes, have a clearer mechanism of VEGF up-regulation via the activation of STAT3[214,215]. Interestingly, STAT3 is heavily involved in the actions of VEGF in diabetic retinopathy, especially since the actions of VEGF on endothelial cells are sufficient to cause STAT3 activation, promoting autocrine VEGF expression[216,217]. STAT3 has also been demonstrated as being critical for HIF1-α activation, and hence VEGF expression in multiple signalling pathways[218]. When one further considers that the actions of hyperglycaemia are sufficient to activate STAT3, via NADPH oxidase activity[219,215], it is clear that STAT3 is a transcription factor of extreme interest in the context of diabetic retinopathy.

As mentioned previously VEGF activates a wide range of signalling cascades in the progression of diabetic retinopathy, of which some of the most important are outlined below. VEGF strongly stimulates vascular inflammation, up-regulating ICAM-1 and MCP-1[220,221,222], and hence VEGF levels are closely linked to the prevalence of leukostasis in diabetic retinopathy, local changes in inflammation, and potentially blood flow if sufficient leukocytes are recruited to block the vessel. VEGF levels in the eye are closely correlated with the permeability of the retinal vasculature and hence the degree of vascular leakage[223,224,214,207]. This is highlighted by observations that anti-VEGF treatments both in animal models and patients can reduce vascular leakage

[205,206,207,201,203,204]. VEGF induces endothelial permeability through an increase in transcellular transport and the formation of fenestrae[225] or caveolin-coated vesicles [226,227], whilst other actions of VEGF are mediated through an increase in paracellular permeability[228,227]. One such example of this is the action of VEGF on plasmin activation via up-regulation of receptors for plasminogen activators[229]. This up-regulation in plasminogen activator receptor leads to increased plasmin and therefore degradation of the subcellular matrix disrupting cell-cell and cell-substrate attachments, hence increasing vascular leakage. This process can also lead to the degredation of the basement membrane, through the actions of plasmin and matrix metalloproteinases, setting the stage for neovascularisation to occur. A further mechanism of VEGF induced permeability centres around the adherens junction protein VE-cadherin. In work by Gavard & Gutkind (2006), immortalised mouse endothelial cells were shown to endocytose VE-cadherin from the surface of the endothelium into early endosomes in response to VEGF[104]. This internalisation was shown to be transmitted via the VEGFR2 receptor in a Src, Vav2, Rac and PAK dependent signalling cascade, occurring as early as 2 minutes post VEGF stimulation. The actions of VEGF in this system were shown to enhance the permeability of the endothelial layer to FITC conjugated dextran, and could be modulated by the specific knockdown of Src or over-expression of constitutively active Src, decreasing and increasing respectively the permeability response to VEGF[104].

Although it is known that hyperglycaemia is key to the development of diabetic retinopathy, a central mechanism for the instigation of the condition has yet to be elucidated. Regardless of this there are known risk factors for the development of the disease, some of which are outlined below.

Inadequate control of blood glucose is one of the major risk factors in the development and progression of diabetic retinopathy[230]. In accordance with this there is also a strong direct link between the development and severity of diabetic retinopathy, and time post-diagnosis of diabetes[231]. In one study the presence of significant levels of protein in the urine (proteinuria), a symptom of diabetic nephropathy, was associated with a 95% increased risk of developing diabetic retinopathy amongst individuals with type 1 diabetes[231, 232]. Hypertension has also been linked to the progression of both diabetic retinopathy and diabetic macular oedema, since it has been shown that tight blood pressure control can reduce the progression of diabetic retinopathy with a 47% reduced risk of deterioration of visual acuity[233]. Other factors that influence the incidence and progression of diabetic retinopathy include serum lipid content, anaemia,

age, increased body mass index (BMI)[234] and pregnancy; in that pregnant women with type 1 diabetes are twice as likely to develop diabetic retinopathy than diabetic women who are not pregnant.

Current data also suggest that diabetic retinopathy may have a genetic component, but studies into the genetic risk factors of diabetic retinopathy are limited by differences in approach that makes comparability between investigations problematic. These data should not however be disregarded, as there are genes that consistently emerge and which are probably significant despite these limitations. Examples of these include aldose reductase, b3 adrenoreceptor, endothelial constitutive eNOS, α-2 integrin, PON-1 (paraoxonase 1), GLUT-1, PAI-1, human leu-cociteantigen-DR and DQ and ACE (angiotensin converting enzyme)[235,236].

There are many treatments in various stages of testing for diabetic retinopathy, such as protein kinase C inhibitors[237], triamcinolone acetonide, inhibitors of the angiotensin pathway[238], growth hormone inhibitors[239,240], antioxidants[241] and cyclooxygenase-2 inhibitors. The best treatment currently available, as with most diabetic complications, is the strict control of glycaemia. This does however cause issues for sufferers as the potential for hypoglycaemia can increase if too much control is imposed, and some weight gain can be expected, increasing BMI and hence another risk factor. Interestingly, it has been shown that tight control of glycaemia was associated with an early worsening of diabetic retinopathy in a third of patients, often going from a mild to moderate stage[242]. Mechanisms for this effect were not clearly defined but hypotheses include changes in blood flow with subsequent hypoxia[243] or increase in shear stress[244], and the actions of exogenous insulin on IGF-1 (insulin like growth factor 1) production potentially facilitating angiogenesis directly[245] or via VEGF [246]. Regardless of these observations, good metabolic control is central to the successful management of diabetic retinopathy, since uncontrolled diabetes will invariably lead to the disease. Another current therapy is tight control of blood pressure, which in itself is a risk factor for diabetes. High blood pressure is thought to increase the levels of shear stress on the endothelial cells, leading to stretching of the vasculature and subsequent VEGF release. Unlike glycaemic control, tight regulation of blood pressure has not been shown to have adverse effects on the progression of diabetic retinopathy and current targets for blood pressure in diabetic patients are below 130/85 mm Hg.

Subsequent to these treatments, photo-coagulation (also called photodynamic therapy) is the remaining therapeutic option for diabetic retinopathy. The treatment involves the use of a laser to coagulate damaged vessels, but this can also lead to local

collateral destruction of neurons. The frequency of the light used seems irrelevant as lasers at 514nm, 532nm, 647nm, 810nm and 560nm-640nm are all effective[247]. Candidates for focal or grid laser photo-coagulation include those with clinically significant focal macular oedema, with the aim being to destroy leaking aneurysms, whereas treatment of clinically significant diffuse macular oedema with laser photo-coagulation is not advised since it is usually unresponsive[248,249,250]. Patients with proliferative diabetic retinopathy are usually indicated for panretinal treatment, with the aim being to ablate the ischemic areas of the peripheral retina, such that the angiogenic factors that cause further progression of the disease are reduced. In recorded trials, pan retinal photo-coagulation reduced the chance of severe vision loss by 50% with focal or grid treatments reducing the risk of vision loss by at least 50% [251]. In some cases patients with severe or very severe non-proliferative diabetic retinopathy are suggested for pan-retinal laser photo-coagulation. These are cases where there is evidence of rapid advancement of the condition, history of poor patient follow up, close relations with diabetes or in patients who have had diabetes for an extended period of time. When photo-coagulation is not successful or there is a non-clearing vitreous haemorrhage or risk of retinal detachment, a further surgical intervention, vitrectomy, may be employed. The main aims of vitrectomy are to remove any opacities obscuring vision, relieve tractional adhesions and manage any complications of previous vitrectomy. However, the procedure is drastic in nature and can cause numerous complications such as retinal detachment, and involves the radical insertion of surgical instruments into the eye. It is however beneficial in patients with severe vision loss due to proliferative diabetic retinopathy, with studies demonstrating that in 25% of cases vision can remain improved at two years post surgery, in comparision to only 15% of those patients who only received careful monitoring[252,253,254,255,256]. Vitrectomy can also be employed to treat macular oedema in cases where focal laser treatment is repeatedly unsuccessful, and has shown promise in restoring vision[257,258,259, 259, 260].

It is often the case in diabetic patients, especially those without good glycaemic control, that they will be affected by more than one consequence of diabetic microangiopathy. As with diabetic retinopathy, diabetic nephropathy describes the damage that can occur to the kidneys as a result of diabetes, caused by dysfunction of the endothelium and the supporting milieu of cells.

1.5 Diabetic nephropathy

In the USA diabetic nephropathy is the most common cause of end-stage renal disease where dialysis is required[261], and is the leading cause of end-stage renal disease worldwide[262]. Between 20-40% of people with diabetes develop nephropathy, with 80% of untreated type-1 diabetics and 20-40% type-2 diabetics progressing to the overt stages of the disease within 15 years[263]. Progression of the disease involves many different cell types, and has distinct phases of development. Changes to the function of the nephron at the level of the glomerulus, such as hyperperfusion and hyperfiltration occur before any clinical changes can be detected. Subsequently the basement membrane of the glomeruli thickens and mesangial cells proliferate, with increased production of extracellular matrix. Microalbuminuria, or the increased presence of albumin in the urine, usually presents early in the disease and is defined as the excretion of greater than 30mg but less than 300mg albumin in the urine per day. Further progression to later stages of the disease is variable[264], indicating that more factors than hyperglycaemia alone influence kidney deterioration.

Many of the biochemical pathways responsible for the diabetic insult are similar to those outlined earlier in endothelial cells with increased flux through the polyol pathway along with increased AGE, active PKC isoforms and hexosamine pathway activity. Interestingly, unlike diabetic retinopathy the influence of genetics in the progression of diabetic nephropathy is thought to play a major role since, as stated previously 20-40% of diabetics progress to the later stages of disease[254], irrespective of glycaemic control. To illustrate how genetics play a role in this disease one study in Pima Indians demonstrated that in individuals who had a history of two successive generations of type 2 diabetes, the incidence of nephropathy was 14% if neither parent had proteinuria, an advanced symptom of diabetes. However if one parent had proteinuria the rate increased to 23%, and to 46% if both parents had proteinuria[265]. So far there have been two methods of collecting genetic data, namely case-control association studies and family studies. Association studies have been the most common approach to date, with genes such as the angiotensin II receptor, extracellular matrix components, various cytokines and proteins involved in glucose or lipid metabolism identified. The approach is not without limitations since some contradictory evidence has been highlighted[266]. However problems also exist in family studies, since there is no simple Mendelian inheritance model available as most affected parents of the patients are dead due to increased morbidity. Investigators have instead used sibling pairs, and

have identified regions on chromosomes 3, 7, 9, 10, 12 and 20[267,268,269] that are of potential interest.

As mentioned previously the early signs of diabetic nephropathy often escape

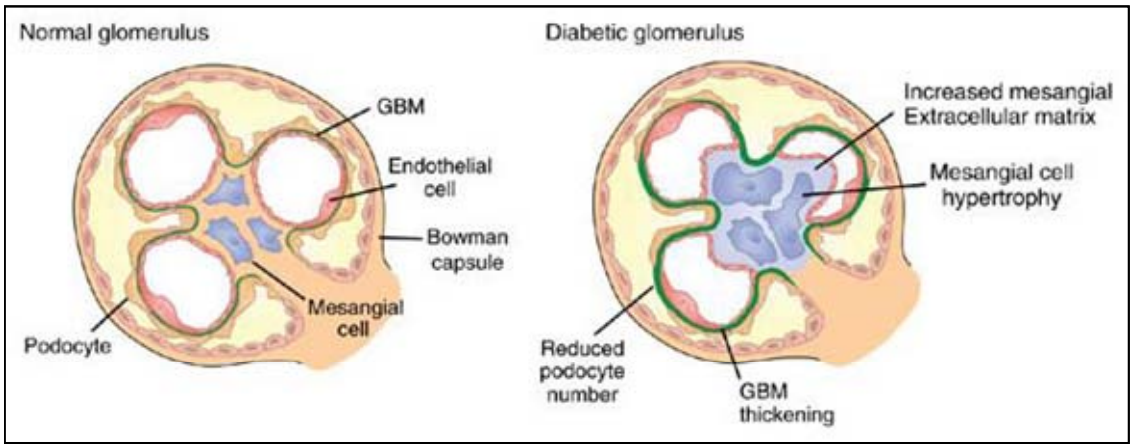

Fig 1.3 Glomerular changes indicative of diabetic nephropathy.
Various changes occur in the glomerulus in response to diabetes including podocyte death, basement membrane thickening and mesangial matrix expansion. Image adapted from Jefferson, J.A. *et al* (2008) Proteinuria in diabetic kidney disease: A mechanistic viewpoint. *Kidney international*. **74** p22-36.

clinical observation and are due to hyperperfusion and hyperfiltration of the glomerulus. These are mediated by constrictions of the afferent and efferent arterioles of the glomerulus, with the afferent more affected, thereby increasing intraglomerular pressure. Many different factors have been identified in the progression of this dysfunction including NO, VEGF, TGF-β, prostanoids and angiotensin II. These early changes in the hemodynamic of the glomerulus facilitate albumin leakage, thickening of the basement membrane and mesangial matrix expansion that provide the early indicators of disease[270,271].

Interestingly all three of the above indicators of the disease can be linked to damage to a specific cell in the kidney glomerulus, namely the podocyte. These are unique, terminally differentiated cells that are located between the glomerular capillary basement membrane and Bowman's space. One of their key features is the podocytic foot process that connects the cell body of the podocyte to the glomerular basement membrane. Combined with the fenestrated endothelium and glomerular basement membrane, podocytes form the filtration barrier that allows water and solutes to pass, but retains proteins inside the capillary. The podocyte foot process is known to be enriched in actin and it's associated proteins, providing the interaction between the foot process and the basement membrane[272]. Another unique characteristic of podocytes is the junction between foot processes, known as the slit diaphragm, which contains proteins such as nephron and podocin. The correct functioning of the podocyte is known

to be important for the functionality of the glomerular filtration barrier, but is also important for counteracting increases in glomerular pressure, as well as the synthesis and maintenance of the glomerular basement membrane and production of VEGF. It is hypothesised by many groups that changes in both the unique structures present in the podocyte and their interactions with other cells can link many of the symptoms of diabetic nephropathy.

In terms of the microalbuminuria that eventually progresses to proteinuria, both the haemodynamic changes and structural changes to the basement membrane and podocytic foot processes are thought to play a role. Changes to podocytic foot processes usually constitute effacement to the basement membrane, a well described finding in diabetic nephropathy, and can be the result of disruption to the slit diaphragm, alterations in the glomerular basement membrane or the interaction between the podocyte and the basement membrane. There are a number of investigations that have proposed mechanisms for podocyte foot effacement[273], ZO-1 has been shown to mis-localise in the presence of hyperglycaemia, from the membrane to the cytoplasm, potentially causing disruption of tight junctions. Signalling pathways such as the MAP kinase pathway have also been suggested to play a role, since activation of the pathway can subsequently activate a small heat shock protein HSP25[274], that is important in the maintenance of the actin cytoskeleton in response to metabolic stress. In fact, investigators have shown that early in chemically-induced diabetes early activation of the MAP kinase pathway is associated with increased activation of HSP25 and preservation of the podocytic foot process structure and maintenance of normoalbuminuria. The actin cytoskeleton of the foot process itself can also become altered since hyperglycaemia has been shown to alter the subcellular localisation and levels of one of the proteins important in active cytosketeton crosslinking, namely α-actinin-4[275].

A further way in which podocytes are involved in the pathogenesis of diabetic nephropathy is through apoptosis. Due to their terminally differentiated nature podocytes cannot regenerate, and as such their loss through apoptosis is thought to permanently alter the characteristics of the filtration barrier. Studies have revealed that the number of podocytes are reduced in both type 1 and type 2 diabetes[276,277], and that apoptosis of podocytes can have a direct effect on the glomerular capillary[278]. Two mechanisms of podocyte apoptosis include the action of ROS directly on podocytes, supported by *in vitro* and *in vivo* experiments, and the observation that antioxidant therapy prevents podocyte apoptosis[279]. Second is the action of angiotensin II through

the renin-angiotensin system, supported by observations in *ex vivo* experiments, an effect that may be mediated through TGF-β activity. Although the podocyte plays a key role in the development of diabetic nephropathy, there are further factors involved including angiotensin II (Ang II), VEGF and TGF-β.

The role of Ang II and the renin-angiotensin system is well known in diabetic nephropathy and its pathological activation is thought to be induced by the hyperglycaemic induction of increased intraglomerular pressure resulting in the production of stretch-stress responses. Ang II is produced initially from the actions of renin on angiotensinogen to produce Ang I (Angiotensin I), then through the actions of angiotensin converting enzyme (ACE) it is converted to Ang II. The majority of the effects of Ang II are mediated through the angiotensin II receptor – type 1 (AT_1) receptor[280,281,282, 283], though a second receptor AT_2 has also been described, and is thought to be important in development. Ang II binding to the AT_1 receptor can have various effects, including vasoconstriction through modulation of NO and intracellular calcium, inflammation via up regulation of NF-κB activity, cell growth, cell proliferation and a decrease in renal blood flow. Due to this, various inhibitors of the angiotensin system have been used in the treatment of diabetic nephropathy, with one such example being synthetic antagonists. Their use in diabetic nephropathy has been reported to decrease proteinuria, TGF-β levels, oxidative stress, pore size of the glomerular membrane, and preserve the structure of the glomerulus and tubules, hence making such drugs important in the prevention of kidney failure in diabetic nephropathy. Untreated however, Ang II activation can up-regulate various cytokines, including VEGF and TGF-β, that have additional effects on the progression of diabetic nephropathy.

VEGF secreted from the podocyte is known to be required for the correct functioning of the endothelium in the glomerulus[284]. In addition, diabetes is known to affect the levels of VEGF in many of its associated disorders, and diabetic nephropathy is no exception, there is however some controversy over whether VEGF levels increase or decrease in response to diabetic nephropathy. The majority of the evidence indicates that VEGF levels and activity increase during disease[285-287], however investigators have also found that the levels of VEGF mRNA in glomeruli of diabetic patients are reduced[288]. One potential explanation for this is the apoptosis and loss of podocytes[289], and as such the true setting of the data is of yet unknown. In terms of the up-regulation of VEGF activity, the stretch response as well as the actions of Ang II[290] are known to up-regulate the levels of VEGF in the context of diabetes with Ang II also capable of

VEGF up-regulation in normoglycaemia[290], potentially through the induction of another inducer of VEGF, HIF-1α[119]. Once VEGF is present it has multiple effects such as an increase in permeability of the endothelium[119], most likely through the actions of NO, down-regulation of the slit diaphragm protein nephron[119], and increased basement membrane thickening by increasing levels of α3 collagen. Investigations into the neutralisation of VEGF in animal models have demonstrated that microalbuminuria can be attenuated[291], with elements of the mesenchymal matrix expansion also impeded[291,292].

TGF-β has been shown in various studies to play an important role in the pathogenesis of diabetic nephropathy. Investigations both in animal models of diabetes[293] and human patients[294] over the past 15 years have demonstrated that TGF-β levels and activity are increased during disease. Evidence of the close link between TGF-β and the progression of diabetic nephropathy comes from a number of observations, including that increased levels of TGF-β in the urine are closely linked with the levels of microalbuminuria. TGF-β signalling, in the context of diabetic nephropathy, has been demonstrated in both *in vitro* and *in vivo* models and is thought to occur through the actions of Smad 3 binding to the promoter regions of TGF-β target genes such as collagen α1 and fibronectin. TGF-β activity can also be communicated through the actions of the MAP kinase pathway[295], which in turn induces the activity of AP-1 (activating protein 1) which is capable of binding and activating Smad proteins involved in the TGF-β pathway. The actions of TGF-β in the context of diabetic nephropathy have earned its status as the major cytokine involved in the mesenchymal matrix expansion[296,297,298,299]. However TGF-β also signals via other molecules to produce various effects such as the induction of connective tissue growth factor (CTGF)[300] and NO production; seemingly through the up-regulation of eNOS[301]. Central to the activation of TGF-β in the context of diabetic nephropathy is the observation that hyperglycaemia increases the level of GLUT-1 in mesangial cells[302] thereby leading to an increase in the transcription rate of TGF-β mainly through the upstream transcription factor (USF) family[303]. However TGF-β has also been shown to be activated through RAGE signalling[119] and by hyperglycaemia or increased levels of albumin *in vitro*. Therapies aimed at reducing the action of TGF-β have been explored and neutralising antibodies have been shown to prevent renal hypertrophy, mesenchymal matrix expansion, increases in mRNA of collagen and fibronectin, GLUT-1 overexpression in mesangial cells and the deterioration of renal function in diabetic mice[304], some of which can also be achieved by the inhibition or down

regulation of Smad3[305]. In addition, it has also been observed that inhibition of the actions of Ang II can lead to lower TGF-β activity[306], postulating a potential link between TGF-β and hypertension.

1.6 The annexins and annexin 2

Since diabetic nephropathy and diabetic retinopathy are diseases of the microvasculature and their associated cells it is important to identify key proteins that contribute to and mediate the deleterious effects of hyperglycemia. One such family of proteins that could fulfil this requirement are the annexins, a group of calcium dependent phospholipid binding proteins found in almost all cells of the body. As a family they share a common C-terminal core domain that contains the calcium-binding sites and which forms a conserved curved disk shape consisting of 4 homologous 'annexin' repeats of around 70 amino acids each. The convex surface contains the calcium binding sites, and it is thought to be the calcium ion bound to the annexin core that is responsible for organising the interaction between the carbonyl and carboxyl groups of the annexin protein and phospholipid membrane respectively[307]. Since the core domain of the annexin family is highly conserved it is the N-terminal domain that largely distinguishes one annexin from another. The N-terminal domain forms a functionally independent subunit and is located adjacent to the convex phospholipid binding face of the C-terminal core. These data came from the elucidation of the crystal structure of annexin 1, where this model of the N-terminal domain was confirmed. Interestingly, it was also shown that in the absence of calcium the N-terminal domain was buried into the C-terminal core[308] hence demonstrating a further reliance of the annexins on calcium for their activation.

Although there are 12 annexins in the vertebrate gene family, in terms of endothelial cell function, annexin 2 has been the most studied. As with all annexins it comprises a C-terminal core domain and N-terminal domain, which is the target of many interactions and modifications that regulate annexin 2 function. One of the most pivotal interactions is the formation of the annexin 2 heterotetramer through binding of two annexin 2 monomers to a dimer of S100A10, a member of the S100 family of EF-Hand proteins[309], also known as the 'annexin 2 light chain'. S100A10[263, 310] was initially identified in a complex with annexin 2[311,312,313]. Like annexin 2, it is found in many different cells and tissues, with the highest expression in the intestine, lung and kidney[314,315]. S100A10 is unique within its gene family in that its actions are

independent of calcium. In other S100 proteins binding of calcium causes conformational changes that expose their hydrophobic surfaces allowing interactions to occur with their various target proteins[310,316,317,318]. However, S100A10 has changes to amino-acids in its EF-hand loops that cause the calcium binding sites to become inactive[312,319], and leave the protein in a permanently activated configuration[309].

Annexin 2 has many intracellular functions, which include its long establised role as a phospholipid binding protein, and more recently its role in actin dynamics. It has been known since 1984 that annexin 2 interacts with actin[311] and since 1994 that annexin 2 can bundle actin monomers into filaments *in vitro* in a calcium dependent manner[320], with the actin binding site of annexin 2 localised to a specific site in the C-terminus by Filipenko & Waisman in 2001[321]. A role for the binding of annexin 2 to actin has been shown in many of its actions, including the secretory pathway and phagocytosis. A further role revealed in a study of live cells transfected with annexin 2-GFP using evanescent field microscopy where it was observed to be enriched in the actin tails of macropinocytic vesicles, an interaction that could be abolished with the transfection of a dominant negative variant of annexin 2[322]. A further recent development in the study of annexin 2 and actin has come from work by Hayes *et al* where it was shown that not only is annexin 2 localised to dynamic actin protrusions inside the cell, but depletion of annexin 2 by siRNA can abolish these structures and cause the cell to lose its protrusive and retractile activity[323]. In addition, it was shown that in specific circumstances annexin 2 can inhibit actin polymerisation at the barbed ends of the actin filament[323]. Links between annexin 2 and actin have been further substantiated with the role of annexin 2 in vesicle rocketing demonstrated in Lowe syndrome fibroblasts, where these structures originate without stimulation and which are decreased with depletion of annexin 2 by siRNA[324]. The actin bundling function of annexin 2 has also been demonstrated physiologically in cells infected by enterohemorrhagic Escherichia coli (EHEC)[325], whilst its identification as a regulator and effector of v-Src induced transformation demonstrates further support for its role in the modulation of actin dynamics[326].

In addition to these 'pathological' settings, annexin 2 function has also been highlighted in a more physiological process, namely wound healing. Epithelial cell migration to heal wounds is known to require actin dynamics, and in a study by Babbin *et al* annexin 2 siRNA treatment caused reduced cell spreading and wound closure, associated with decreased formation of filamentous actin along the base of the cells[327]. Interestingly, the dynamics of Rho were also examined in this system, and upon

treatment with annexin 2 siRNA, Rho was found to be dissociated from the plasma membrane and less active. Further to this, transfection of constitutively active Rho restored the ability of epithelial cells to close wounds demonstrating the importance of actin dynamics and the role of annexin 2 in wound healing.

As mentioned previously, annexins are phospholipid-binding proteins. In the case of annexin 2, binding to phosphatidylinositol (4,5)-bisphosphate (PIP_2) has been described in various settings including infection by enteropathogenic Escherichia coli, where binding of annexin 2 to PIP_2 causes actin accumulation[328]. The translocation of annexin 2 to membranes has been shown to be responsive to both calcium[329], pH[330] and phosphorylation at the N-terminus[331]. When taken together with its ability to interact with actin, the phospholipid binding properties of annexin 2 are central to its role in many different cellular processes, some of which will be outlined further below.

During endocytosis the transport and fusion of endocytic vesicles is mediated by various proteins, one of which is annexin 2. Assays on the fusion efficiency of early endosomes revealed that normal endosomes could fuse with good efficiency, but that when endosomes were sonicated, and hence fractionated into smaller endosomes, only some of them could fuse[332]. By examining which proteins were transferred to a donor endosome it was possible to identify proteins necessary for endosome fusion, one of which was found to be annexin 2. Many subsequent studies have highlighted the role of annexin 2 in the endocytic pathway. For example, it was shown that binding of annexin 2 to cholesterol in endosomes can mediate their interaction with the cytoskeleton[333,334]. It was also shown that when cholesterol was depleted from endosomes, annexin 2 could no longer be isolated from them[333]. In addition to this, more recent publications have demonstrated the role for annexin 2 in the generation of early endosomes where it has been shown as being important for nucleating actin on their surface in association with Spire1 and Arp2/3[335], with phosphorylation of annexin 2 shown to be essential for its proper endosomal association and facilitation of the endocytic pathways[336].

Annexin 2 is also involved in the secretion of molecules by the cell, studies using adrenal chromaffin cells showed that annexin 2 is associated with secretory granule membranes and loss of secretory activity of permeabilised cells, due to leeching of intracellular proteins, can be rescued by the addition of annexin 2, a process that is then reversible by the addition of an antibody directed against annexin 2[337,338]. In addition, annexin 2 has been shown to be critical for regulated secretion of molecules from endothelial cells when in complex with S100A10, since disruption of this interaction reduces secretion from these cells[339]. Interestingly, annexin 2 has also been

shown to be selective in its secretory role, since upon its inactivation in endothelial cells, vWF secretion is inhibited whilst that of t-PA is not[340]. Phagocytosis provides another example of a process that requires organised membrane dynamics, and which is therefore likely to involve annexins. Indeed, annexins 1-5 are associated with different stages of phagocytosis, with annexin 2 found on the plasma membrane co-localised with F-actin at membrane protrusions in macrophages[341]. In addition, annexin 2 has been shown to regulate the kinetics of Src and FAK activation in retinal phagocytosis[342].

Junction formation in endothelial and epithelial cells requires the participation of many different proteins. Annexin 2 has been identified in complex with Rac-1, a small GTPase involved in the regulation of cell migration and adhesion in epithelial cells. In a report by Hansen *et al* (2002) an annexin 2:Rac-1 complex was identified at epithelial cell junctions in response to cell-cell contact, a process dependent upon production of specific anionic phospholipids by PI3-Kinase[343]. It is thought that in this context annexin 2 stabilises Rac-1 at the plasma membrane to facilitate junction formation, hence giving annexin 2 a role in epithelial junction formation. Further evidence for a role for annexin 2 in epithelial adherens junction formation has been shown by two separate studies that demonstrated failure of E-cadherin localisation to the periphery upon annexin 2 siRNA treatment[344,345]. In terms of endothelial junction formation it is known that when endothelial cells reach confluence the cholesterol and annexin 2 content of the plasma membrane increases[346]. It is also known that in endothelial cells an annexin 2-SHP-2 complex is recruited to the plasma membrane in response to rising cholesterol levels and cell-cell contacts, leading to de-phosphorylation and stabilisation of adherens junction proteins[347]. More recently, this complex with SHP-2 has been shown to be sensitive to calcium, the actions of growth factors, and heat shock protein 70 (HSP70)[348]. Indeed, a direct link has also been found between annexin 2 and VE-cadherin, the main adherens junction protein in endothelial cells. In a study by Heyraud *et al* annexin 2 was found to bind VE-cadherin in confluent human umbilical vascular endothelial cells (HUVEC), translocating from the cytosol to the plasma membrane. It was also shown that annexin 2 in lipid rafts bound both actin and VE-cadherin, preventing the lateral diffusion of the VE-cadherin complex. Further to this, treatment with siRNA directed against annexin 2 caused mislocalisation of VE-cadherin away from adherens junctions, with VEGF decoupling annexin 2 from VE-cadherin potentially facilitating the switch from a quiescent to an immature state[349]. Taken together, these observations highlight annexin 2 as a potentially important protein in the formation of cell-cell junctions.

As well as having intracellular actions, various extracellular roles have also been identified for annexin 2, despite the fact it lacks a typical signal peptide for secretion. One such interaction of annexin 2 on the surface of cells was described with tenascin-C. In a recent review tenascin-C was reported as having roles in the body's response to stress and trauma, such that the tenascin-C knock-out mouse appears normal until it encounters a pathological challange[350]. The binding of tenascin-C to annexin 2 on the surface of endothelial cells has been shown to cause three responses: loss of focal adhesions, growth of confluent cells exposed to growth factors, and increased cell migration[351]. All these effects were blocked by an antibody specific for annexin 2 prior to the addition of tenascin-C, indicating that the processes are annexin 2 dependent and that the complex formed can signal to the inside of the cell. The physiological significance of the interaction of tenascin-C with annexin 2 has been linked to tumour formation, where endothelial annexin 2 binds to tenascin-C on lymphoma cells[352].

Tenascin-C is not the only protein that has been described to bind extracellular annexin 2, in the development of the foetus 'immunoglobulin G' (IgG) molecules are transferred across the placental membrane by Fc receptors on the membrane surface. Annexin 2 has been shown to have Fc-like properties, such that it can bind IgG and is found in placental tissue. Whether annexin 2 functions as a transport molecule for IgG was the subject of a study conducted by Kistoffersen & Matre, and although tetrameric annexin 2 was found on the surface of the syncytiotrophoblast microvillous plasma membrane it was unclear as to whether annexin 2 functioned to transport the IgG across into the foetal tissue[353]. Another surface annexin 2 interaction has been described in a study conducted by Wright *et al*. In this investigation the human cytomegalovirus was found bound to the surface annexin 2 of endothelial cells, and that antibodies directed against annexin 2 could reduce the infectivity of the virus[354].

The ability of annexin 2 to bind membranes in an intracellular environment is well characterised. In contrast, the ability of annexin 2 to bind membranes when on the surface of endothelial cells was shown in a study by Lee *et al*, where annexin 2 was found to co-localise with other tight junction proteins such as occludin. In this instance it was proposed that it was the tetrameric form of annexin 2 that mediated the formation of the junction by bringing the two membranes close together. To this end it was also shown that a synthetic peptide targeted to disrupt the interaction between S100A10 and annexin 2, therefore inhibiting the tetrameric from of the protein, significantly reduced tight junction formation in these cells[355].

In addition to having roles in homeostatic and normal physiological processes, extracellular annexin 2 has also been implicated in the infectivity of HIV-1. In this instance it is the phosphatidyl serine binding capacity of annexin 2 that is exploited by HIV-1 virus particles to infect macrophages, an effect which can be inhibited using specific inhibitors of annexin 2[356,357]. More recently annexin 2 has been shown to be coerced intracellularly post-infection by HIV-1, where binding of PIP_2 facilitates the HIV-1 gag gene to mediate viral assembly[358].

The most extensively studied extracellular interaction of annexin 2 is its binding to plasminogen and tPA on the surface of endothelial cells to mediate the formation of plasmin[64]. Annexin 2 has been implicated as an important regulator of the fibrinolytic system[359] with evidence for this originating from various studies, including an examination of acute promyelocytic leukaemia (APL), in which patients suffer from haemorrhagic diathesis. In a study by Menell *et al* it was noted that patients with APL had increased amounts of annexin 2 on the surface of their leukemic cells, and an increased formation of plasmin[360]. It was also shown that modulation of annexin 2 levels could influence the rate of plasmin production and hence alter the fibrinolytic potential of the cells[360]. Further evidence for the role of annexin 2 in fibrin generation emerged from studies utilising the annexin 2 knockout mouse, that displays not only reduced tPA-dependent plasmin generation, but also increased fibrin clots and decreased migration of endothelial cells both in *in vitro* and *in vivo*[361]. Regulation of the effect of annexin 2 on plasmin generation is thought to come in part from the actions of its binding partner S100A10, since protein levels of the latter have been linked to the invasiveness of metastatic cancer cells[362], and it regulates the translocation of annexin 2 to the surface of endothelial cells[363]. Roles for the annexin 2 dependent generation of plasmin have also been linked to influenza infection, where annexin 2 incorporated into the viral envelope generates plasmin that degrades haemagglutinin and permits further infection[364].

The study of annexin 2 has most often concentrated on its function inside or on the surface of cells, there are however a few studies on a soluble circulating form of annexin 2. Most of these have implicated soluble annexin 2 as a modulator of the immune system, with the ability to inhibit lymphocyte proliferation in the retroplacental serum[365], possibly to aid protection of the foetus from the maternal host defences. It has subsequently been shown by the same group that IgG and IgM secretion from mononuclear cells can be inhibited by soluble annexin 2[366]. The second setting for the function of soluble annexin 2 originates from studies of osteoclast formation, the cells

responsible for bone resorption. It was observed that increased levels of soluble annexin 2 enhance the differentiation of osteoclasts in an autocrine manner[367]. Another interesting development has arisen more recently where a putative receptor for the annexin 2 protein has been found on osteoblasts and inhibition of this receptor has been shown to inhibit osteoclast formation[368]. This result in itself may prove relevant to other fields of annexin 2 research and may provide more potential roles for soluble annexin 2.

1.7 Annexin 2 and Diabetes

Given the diverse roles of annexin 2, it is unsurprising that it is implicated in many different physiological and pathological pathways. As mentioned earlier, two main pathological consequences of diabetes are diabetic retinopathy and nephropathy, multifactorial complications centred around the persistence of hyperglycaemia. Although annexin 2 has not been directly implicated in the progression of either of these complications of diabetes there are a number of observations regarding its function that indicate that it plays a role.

Firstly, annexin 2 is responsive to hypoxia, translocating to membranes of cells independently of calcium[369]. Since hypoxia is a key element in diabetic retinopathy leading to VEGF production, hyperpermeability and neovascularisation, it would be interesting to further elucidate whether the actions of annexin 2 in hypoxic hyperglycaemic conditions are protective or ultimately destructive. With regard to diabetic nephropathy, annexin 2 levels have been reported to increase in acute renal failure[370], and annexin 2 has been demonstrated to be important in the trafficking of aquaporin 2, a critical membrane protein of the kidney[371,372,373], that is important in the osmotic permeability of the collecting duct of the nephron. Of particular interest is the work carried out by Ishii *et al* where it was shown that addition of recombinant annexin 2 to a murine model of type-2 diabetes was sufficient to protect against the development of albuminuria and histological changes[374].

Although there have been a number of descriptions of annexins facilitating the functions of cell signalling pathways important in diabetes, some of the more complete and tangible data regarding a potential role for annexins in diabetes have emerged by examining the role of annexin 2 in the formation of plasmin. As stated previously, in normal conditions is it known that annexin 2 on the surface of endothelial cells can bind both plasminogen and tPA to facilitate plasmin formation and hence increase

fibrinolysis. Diabetes has long been known to induce a hypercoagulable state, and it is currently thought that a change in the ability of annexin 2 to perform it's role in the formation of plasmin could play some part in this. Some of the initial data in this regard came from observations that annexin 2 is an early non-enzymatic glycation product[375], and that in cultured primary endothelial cells grown in high glucose, and high insulin, plasmin formation is reduced, an effect that can be reversed with the addition of recombinant annexin 2[376]. These observations led to the hypothesis that glycation of annexin 2 is sufficient to disrupt its ability to function in the generation of plasmin[377], although this has not been shown experimentally.

Further understanding of the effects of high glucose on annexin 2 came from a study by Lei *et al* who showed that stimulation of endothelial cells with high glucose was sufficient to enhance binding of annexin 2 to the heat shock protein HSP90α. These authors also demonstrated that this binding resulted in increased translocation of annexin 2 to the surface of endothelial cells, and that as a result an increase in plasmin production was observed[378], contrary to the findings of Ishii *et al*[376]. The major distinction between the two studies was the addition of insulin, since in both studies high glucose was used, but only Ishii *et al* additionally exposed the endothelial cells to high insulin. The actions of insulin on annexin 2 have been investigated and it is known that insulin stimulation of cells leads to an increase in tyrosine phosphorylation of annexin 2[379,380], which in turn drives changes in the actin cytoskeleton[380]. The question of whether this phosphorylation event is sufficient to perturb tPA or plasminogen binding to annexin 2 has yet to be answered. There is however evidence to suggest that other modifications of annexin 2 can alter its binding capacity for target proteins. One example of this is the action of hyperhomocysteinaemia on annexin 2, a condition known to influence diabetic retinopathy[381]. The presence of elevated levels of homocysteine have been shown to reduce the cell surface binding of tPA by 60%, and this has been attributed to the change of the Cys8 residue on the N-terminus of annexin 2 to a HomoCys8 which does not favor tPA binding[382,359,383]. By examining the evidence so far one might therefore speculate that phosphorylation of annexin 2 could modify its ability to act as a plasminogen/tPA receptor, especially since the tyrosine phosphorylation site of annexin 2 is only 15 residues away on Tyr23[384]. This presents the interesting question of whether these differences in annexin 2 function would arise in both type-1 and type-2 diabetics which differ with regard to the presence or absence of insulin.

Finally, because annexin 2 is implicated in the maintenance of endothelial junctions as outlined earlier, through interactions with SHP-2 and stabilisation of VE-cadherin, the question arises as to whether phosphorylation, incorporation of homocysteine or glycation are sufficient to affect the function of annexin 2 in this context. It would be interesting to know whether inactivation of annexin 2 though the action of diabetes could lead to destabilisation of the endothelial cell junctions, resulting in the leakage of fluid seen in diabetic retinopathy, and macular oedema.

Having outlined a number of potential roles for annexin 2 in the progression of diabetes, this thesis aims to examine whether annexin 2 function is altered by the action of diabetes, or whether loss of annexin 2 is sufficient to alter the progression of diabetic microvasculopathies. These questions will be addressed using both *in vitro* endothelial cell culture models, and by chemical induiction of diabetes in the annexin 2 knock-out mouse.

Chapter 2 Materials and Methods

Chapter 2 Materials and Methods

2.1 Materials

Mouse anti-annexin 2 was purchased from BD Biosciences and anti-mouse-HRP, anti-rabbit-HRP, and rabbit-anti-GFAP purchased from DakoCytomation. Rabbit anti-GLUT-1 was purchased from Millipore, and mouse anti-transferrin receptor purchased from Invitrogen. Phalloidin-633, anti-mouse alexafluor-488, anti-rabbit alexafluor-568 and anti-goat alexafluor-488 were all purchased from Molecular Probes, with mouse anti-VE-cadherin antibody purchased from Santa Cruz. DAPI along with collagen I, collagen IV and fibronectin, in addition to Percoll™ and streptozotocin were purchased from Sigma. F-10, M199 and α-MEM basal media systems together with penicillin / stretptomycin and FCS (foetal calf serum) were purchased from Invitrogen whilst the EBM2-MV culture media was purchased from Lonza with all other additives from Sigma.

All western blotting reagents were obtained from BioRad, and all histological reagents obtained from Agar Scientific ltd, with the exception of OCT (optimal cutting temperature) that was obtained from RA Lamb. Optium plus blood glucose testing strips were purchased from Abbott whilst all anaesthetics were obtained through the named veterinary surgeon with fluorescein obtained from Sigma. Collagenase and dispase were obtained from Worthington biochemical and recombinant mouse VEGF-164 purchased from R&D systems. Finally, mouse anti-tubulin (clone ID: 1A2) and Rabbit anti-ZO-1 were kindly supplied by Prof Karl Matter (Inst of Ophth, UCL). Mouse anti-annexin 2 (clone ID: HH7) was kindly supplied by Prof Volker Gerke (Inst Med Biochem, University of Münster) and mouse anti-annexin 2 (clone 444) kindly supplied by Jesus Ayala-Sanmartin (UPMC, Paris). HUVEC were supplied at passage one by Prof Daniel Cutler (LMCB, UCL), whilst GPNT and hCMEC/D3 were both supplied by Prof John Greenwood (Inst of Ophth, UCL).

2.2 Primary cell and immortalised cell lines

2.2.1 Cell culture

For those cells in frozen stocks an aliquot was quickly defrosted at 37ºC, resuspended in 10 ml fresh media and centrifuged at 200 x g for 5 min at which point

the supernatant was discarded and the cells diluted to an appropriate concentration for culture. All cells were maintained in a humidified atmosphere at a constant temperature of 37ºC and 5% CO_2, although each cell line required a different media composition and culture substratum (Table 1).

Cell Line	Media	Substratum
GPNT	1:1 F-10 / α-MEM, 10% FCS, P/S*	Collagen I
HUVEC	M199, 20% FCS, 50µg/ml Gentamycin, 30µg/ml ECGS	Gelatin
hCMEC/D3	EGM2-MV, 12.5% FCS, 25% supplied additives	Collagen I
Primary murine endothelial cells	EBM-2, 16% PDS, 50µg/ml puromycin^, P/S*	Collagen IV + Fibronectin

*P/S = Penicillin 100U/ml + Streptomycin 100µg/ml
^= Removed 48 hours post isolation

Table 1: Media composition and culture substratum of cell lines used in this thesis.

The substratum was prepared prior to plating the cells by addition of sufficient volume of coating solution to cover the surface followed by removal and washing of the surface (Table 2).

Cell Line	Substratum	Concentration	Incubation time	Incubation temp	Wash*
GPNT	Collagen I	40 µg/ml	o/n	37ºC	HBSS
HUVEC	Gelatin	1% w/v	10 min	RT	N/A
hCMEC/D3	Collagen I	40 µg/ml	o/n	37ºC	Water
Primary murine endothelial cells	Collagen IV Fibronectin	100 µg/ml 50 µg/ml	o/n	37ºC	Water

* = All washes repeated twice

Table 2: Coating solution concentrations and coating protocols for the different cell lines used in this thesis.

With the exception of the primary murine endothelial cells, once 70-90% confluence was achieved, cells were passaged by washing once in PBS, followed by incubation in 0.2% trypsin until cells had loosened. Cells were then collected and mixed with at least 2 volumes media, after which they were centrifuged at 200 x g for 10 min, diluted to the appropriate concentration and plated as required.

2.2.2 Primary isolation of endothelial cells from murine brain

Wild type or annexin 2 null mice were sacrificed by asphyxiation in CO_2 followed by cervical dislocation. Brains were then harvested, and the cerebellum, meninges and white matter removed. The remaining grey matter was homogenised using 10 passes of a Dounce homogeniser, centrifuged at 400 x g for 10 min and the supernatant discarded. The pellet was then fully resuspended in 22% bovine serum albumin solution in PBS and centrifuged at 1200 x g for 20 min. Once complete the myelin plug clearly visible at the top of the tube was removed without disturbing the

pellet and transferred to a fresh centrifuge tube. The pellet was washed once in working buffer (HBSS -$CaCl_2$, -$MgCl_2$, -$MgSO_4$, 0.5% w/v BSA, 10mM HEPES, 100u/ml penicillin, 100µm/ml streptomycin) and the pellet fully re-suspended and centrifuged at 400 x g for 10 min; followed by storage at 4°C until required. Fresh 22% bovine serum albumin in PBS was added to the myelin plug from the previous step followed by centrifugation as previously described to yield three further pellets. The remaining myelin plug was then discarded and the four pellets pooled, centrifuged at 400 x g for 10 min, supernatant discarded and pellet re-suspended in 1 ml working buffer. Each sample was then loaded onto a 70 µm nylon mesh (BD biosciences Cat# 352350), and washed thoroughly with working buffer, such that the vessel fragments adhered to the surface and the contaminating waste was allowed to wash through. The nylon mesh containing the vessel fragments was then inverted and washed through to allow the vessel fragments to collect in a fresh centrifuge tube. Vessel fragments were then centrifuged at 400 x g for 10 min and re-suspended in a solution containing 1 mg/ml collagenase and 1 mg/ml dispase. This solution was then incubated at 37°C whilst rotating at 250 rpm to allow efficient enzymatic activity. At the end of the incubation step, the solution was centrifuged at 400 x g for 10 min and the pellet resuspended in the primary cell culture medium. The vessel fragments were then plated onto the selected substratum at the appropriate density to ensure enough cells were present for the experiment required.

2.3 Protein isolation and analysis

2.3.1 Isolation of cell-surface annexin 2

Cells were washed twice in HBSS (1.26mM $CaCl_2$, 493µM $MgCl_2$, 407µM $MgSO_4$) and then incubated for 2 min in a 5 mM EGTA in PBS at 4°C. The EGTA solution was then collected and combined with an equal volume of acetone and left for a further 10 min at 4°C. Solutions were then centrifuged at 13,000 rpm in a tabletop microfuge, supernatants discarded and pellets re-suspended in a reducing sample loading buffer (250mM Tris, 10% glycerol, 2% SDS, 270 mg/ml DTT, 100 mg/ml bromophenol blue, pH 6.8). Samples were heated at 95°C for 5 min, followed by storage at -20ºC until required.

2.3.2 Preparation of whole cell lysates

Cells to be lysed were washed twice in HBSS, followed by lysis in 95°C reducing sample loading buffer. Lysed cells were scraped and transferred into Eppendorf tubes and heated at 95°C for 5 min followed by storage at -20°C until required.

2.3.3 Protein isolation from whole tissue

Tissue to be lysed was first isolated and washed in ice cold PBS, followed by homogenisation in lysis buffer (20 mM Tris, 150 mM $NaCl_2$, 1 mM EDTA, 1 mM EGTA, 2.5 mM $Na_4P_2O_7$, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1% Triton X-100, pH 7.5) containing protease and phosphatase inhibitors (Sigma – Catalogue # P8340). Protein quantification was conducted using a Bradford assay (BioRad), as per the manufacturer's instructions such that an equal amount of protein could be loaded in subsequent analyses.

2.3.4 SDS-PAGE and western blotting

Samples were resolved by 10% SDS-PAGE at 150 V for 90 min and transferred onto PVDF membranes at 400 mA at 4°C. Membranes were blocked with 10% skimmed milk in 0.05% Tween PBS solution for 1 h at room temperature, and then incubated with primary antibody loaded in fresh blocking solution overnight at 4°C. PVDF membranes were then washed several times in 0.05% Tween in PBS followed by the addition of the secondary antibody, conjugated to HRP, in fresh blocking solution at room temperature for 1 h. Bands were resolved using ECL reagents as per the manufacturer's instructions.

2.4 Biochemical and density gradient separation

2.4.1 Detergent based surface / intracellular isolation of annexin 2

Surface annexin 2 was first isolated from cells as detailed earlier (2.3.1), after which cells were then washed once in HBSS and then lysed in 20 mM Tris, 150 mM $NaCl_2$, 1 mM EDTA, 1 mM EGTA, 2.5 mM $Na_4P_2O_7$, 1 mM β-glycerolphosphate, 1

mM Na_3VO_4, pH 7.5 containing 2% Triton-X100, with incubation at 4°C for 2 min. The cell lysates were then collected in Eppendorf tubes and centrifuged at 13,000 rpm for 10 min, and once complete the supernatant was transferred to a fresh Eppendorf tube. An equal volume of acetone was added to the supernatant and the solution was allowed to incubate for 10 min at 4°C. Once the incubation was complete the solution was spun at 13,000 rpm for 10 min. The supernatant was removed and discarded, after which both pellets from the two centrifugation steps were re-suspended in reducing sample buffer and heated to 95°C for 5 min. All samples were then stored at -20°C until required.

2.4.2 Percoll sub-cellular fractionation

Cells were washed twice with PBS followed by incubation in just enough 1x trypsin solution at 37°C to cover the cells. Once the cells were loosened, media containing FCS was added to inactivate the trypsin with the cells collected and centrifuged in a 50 ml centrifuge tube at 200 x g to form a pellet. The supernatant was removed and the cells re-suspended in 1 ml of homogenisation buffer (10 mM HEPES, 200 mM sucrose, 3 mM imidazole, pH7.2) at 4°C. The cells were then passed through a ball-bearing homogeniser with a 10 μm clearance 20 times at 4°C to gently break them open. The resulting cell homogenate was collected and spun at 4000 rpm in a tabletop microfuge at 4°C and the supernatant transferred to a fresh centrifuge tube containing 5 ml of Percoll™ solution (Density: 1.08g/ml). The solution was well mixed, loaded into a 7 ml ultracentrifuge tube and centrifuged at 25,000 x g at 4°C for 45 min. Once complete, the cell lysate solution was fractionated into 1 ml fractions, and 20 μl Triton X-100 and 10 μl 0.5 M EGTA solution added to each fraction and mixed well. Each fraction was then centrifuged in the same rotor at 200,000 x g for 1 h. Once complete, the supernatants were removed from the fractions and an equal volume of acetone was added to each followed by incubation at 4°C for 10 min. Solutions were then centrifuged at 13,000 rpm and pellets re-suspended in reducing sample loading buffer, after which they were heated at 95°C for 5 min and stored at -20°C until required.

2.4.3 Sucrose density gradient sub-cellular fractionation

Cells to be homogenised were trypsinised and centrifuged as for cell passaging, then re-suspended in a homogenisation buffer (10 mM HEPES, 200 mM sucrose, 3 mM imidazole, pH7.2). The cell suspension was then passed 20 times through a ball-bearing

homogeniser set with a 10 μm clearance. The cell homogenate was centrifuged at 4000 rpm at 4°C to remove whole cells and nuclei. The supernatant was removed, adjusted to 1 ml and loaded on top of a discontinuous gradient consisting of 5 x 1 ml fractions of 60%, 50%, 40%, 30% and 20% sucrose respectively. Samples were then centrifuged at 100,000 x g for 16 h with minimal acceleration and braking. Once complete the sucrose gradient was fractionated into 1 x 250 μl (fraction 1) and 11 x 500 μl (fractions 2-12), to which 800 μl of acetone at 4°C was added and incubated at 4°C for 10 min. Fractions were then centrifuged at 14,000 rpm for 10 min at 4°C, the pellets re-suspended in 1x reducing sample buffer and incubated at 95°C for 5 min prior to storage at -20°C until required.

2.5 Streptozotocin induced diabetes

2.5.1 Streptozotocin induced model of diabetes

Wild type c57bl/6 and annexin 2 null mice (Ling *et al.*, 2004) were maintained on a regular diet of food and water *ad libitum*, in accordance with UK Home Office regulations. When possible, prior to induction of diabetes, mice were fasted for 4 h and their weight and fasting blood glucose levels recorded as detailed below, with mice given 1 week to recover before continuation of the protocol. To induce diabetes mice were first fasted for 4 h followed by an intraperitoneal injection of 55mg/kg streptozotocin in 0.1M citrate buffer (pH 4.5), with sham animals receiving citrate buffer only. This was then repeated over five consecutive days, followed by glucose measurements at 14 days post the first injection, with treatment deemed successful if blood glucose ≥250 mg/dl. Weight and glucose testing were then continued every two weeks for the first two months, followed by measurements every four weeks, either with or without 4 h fasting prior to sampling depending upon the study. In some experiments mice had their weights and blood glucose levels recorded, followed by a 4 h fast, and then a subsequent glucose measurement recorded to assess the contribution of fasting to changes in blood glucose.

2.5.2 Weight and glucose testing

Animals to be examined were placed in a fresh cage on scales suitable for weighing live animals and the weight recorded. Animals were then put back into their

own cage whilst littermates were weighed. Once complete, each mouse in turn was placed in a restraint to allow easy access to the tail, and a small cut made laterally to either of the tail veins. In the first set of experiments, a small drop of blood was collected and tested using 3 Optium plus glucose testing strips measured in an Optium glucose testing machine. As part of the second study, in situations where blood glucose concentrations were >500mg/dl and hence too high for the machine to record, 5 µl of blood was diluted with an equal volume of deionised water, blood glucose readings re-measured and multiplied by two to give the final concentration. Pressure was then applied to the cut on the tail to aid healing, and the mouse returned to the cage to recover, followed by careful monitoring to spot any complications that may arise.

2.5.3 Fluorescein angiography

Mice to be examined were weighed and given anaesthetic; 10 µl per gram body weight of 6.3 mg/ml Ketamine and 0.1 mg/ml Domitor, with signs of life checked regularly. Once sufficiently anaesthetised the mouse was placed in a nose bar, and it's whiskers taped out of the way of the microscope objective. Phenylephrine hydrochloride drops (2.5%) were given to dilate the pupils, and washed off with 0.3% hypromellose once sufficient dilatation had been achieved. The scanning laser ophthalmoscope (SLO) was then positioned and a reflective image of the retina taken. The mouse was then injected with 50 µl 10% fluorescein and fluorescent images taken with the SLO at 15 s intervals. Once 10 min of imaging was complete, additional images were taken as required, and the mouse removed from the nose bar. The mouse was then given an injection of 10 µl per gram 0.1 mg/ml antisedan to reverse the anaesthesia, and signs of life monitored carefully. Once all the sampling was complete, mice were taken to a recovery room where heat, via a heat mat, and soak diet was provided, for 24 h to aid recovery.

2.5.4 Assessment of polydipsia

Water consumption was assessed by measurement of the weight of the drinking water bottle before and after a given 24 h period. Samples were taken on days where the cages were not due to be cleaned to minimise vibration to the cage and hence artifactual loss of water.

2.5.5 Assessment of urinary albumin and creatinine

Urine samples were either taken from live animals at pre-determined time points or at death. For a pre-determined time point, mice were placed over a cage lid underlined with cling film and allowed to urinate. For urine collected at death, the bottom of the carbon dioxide chamber was carefully cleaned, and the mouse placed into the chamber, as per schedule 1 methods. Upon death the urinary sphincter would relax and the urine could be collected from the bottom of the chamber. In addition to this, any remaining urine could be extracted directly from the bladder, using a syringe and needle. Regardless of the collection point, samples were first centrifuged for 1 min to remove any contaminants and then subjected to ELISA for albumin (Exocell, Albuwell M Cat# 1011) and creatinine (Exocell, Creatinine companion Cat # 1012) as per the manufacturer's instructions.

2.6 Embedding, sectioning and digestion of tissue samples

2.6.1 OCT embedding and sectioning

Tissue to be embedded was dissected from the mouse and washed briefly in PBS. Tissue samples were then washed twice in OCT compound, before transfer into a plastic mould of a suitable size for the sample containing fresh OCT. Samples were then cooled using dry ice until the OCT was set, at which point they could be transferred to a -80ºC freezer until required. Upon sectioning, samples were loaded into a Leica CM1850 cyrostat with equipment calibrated as per the manufacturer's instructions, and the sample loaded onto the chuck using fresh OCT as an adhesive. Samples were first trimmed at 20 μm until the sample was visible, at which point serial sections were taken at 10 μm. Sections were transferred onto Superfrost® plus slides and dried for at least 1 h under a fan. At this point slides were either stored at -80ºC or transferred to the staining protocol required.

2.6.2 Wax embedding and sectioning

Samples to be mounted in wax were dissected from mice and immersed immediately in buffered 4% formaldehyde followed by incubation for 24 h at room temperature. Once this fixation step was complete, samples were further dissected as

required, loaded into histological cassettes and placed into a Leica TP 1020 tissue processor for overnight processing as per the manufacturer's instructions. Once the samples were in histological wax they were embedded in fresh wax, using plastic moulds, loaded on top of the histological cassettes used for processing. At this point samples could be kept at room temperature or sectioned using a microtome. When sections were required, excess wax was trimmed from the outside of the histological cassette and the block loaded into the microtome. Samples were first trimmed until an even cutting surface was achieved, and then sections were cut at 6 μm. Sections were loaded onto standard glass slides using a 55ºC water bath to float the sections on, at which point they could be easily transferred onto glass slides, drained, and heated on a hot plate prior to use in the staining protocol required.

2.6.3 EPON™ Resin embedding and sectioning

Eyes to be embedded were prepared with an incision along the ora serrata and removal of the lens. Kidney samples were taken by cutting a <1mm section of the cortex. In all cases samples were first fixed in a solution containing 2% paraformaldehyde (PFA) and 2% glutaraldehyde in 1x PBS for 24 h at room temperature. Samples were then washed 3 times in 0.1 M sodium cacodylate ensuring removal of any glutaraldehyde, and stained in a solution containing 1% osmium tetroxide, and 1.5% potassium ferricyanide for 1-2 h at 4ºC in the dark. When this was complete samples were washed 3 times in 0.1 M sodium cacodylate, again ensuring thorough disposal of any osmium tetroxide, and stained further in a solution containing 1% tannic acid in 0.05 M sodium cacodylate for 40-60 min at room temperature with gentle end over end rotation. Once complete, samples were washed 3 times in 0.05M sodium cacodylate with a final wash in deionised water. Samples were then immersed in 70% ethanol for 10 min, followed by 90% ethanol for 10 min and two immersions in 100% ethanol for 10 min each. At the end of these steps the samples were immersed twice in propylene oxide for 10 min each, after which they were placed in a solution containing 1:1 propylene oxide and EPON™ epoxy resin overnight with gentle end-over-end rotation. Once complete, samples were given two separate immersions in fresh EPON™ for 4 h each, at which point they were transferred into a suitable mould, immersed in fresh EPON™ and incubated at 50ºC overnight or until the EPON™ had hardened. Samples could then be stored at room temperature until required or sectioned. Sectioning was conducted on a Leica ultracut S microtome and samples were first

trimmed with glass knives, followed by production of either semi-thin (0.7 μm) or ultrathin (60 nm) sections which were mounted on slides and transferred to the staining protocol required.

2.6.4 Trypsin digest of retina

Eyes were dissected and placed immediately into 2x PBS at 4ºC. When sampling was complete, eyes were fixed with 2% PFA in 2x PBS for 2 min, and then washed 3 times in 2x PBS. Eyes were then immersed in fresh 2x PBS and dissected under the microscope with a circular cut around the ora serrata removing the front of the eye and the lens. The remaining eye cup was then carefully turned inside out such that the retina could be removed from the eye cup. Once the retina had been isolated it was fixed for 48 h in 4% PFA in 1x PBS at 4ºC. Once complete, retinas were washed in a large volume of PBS for 24 h at room temperature, followed by incubation in 3% trypsin, 0.1 M Tris-HCL (pH 7.8) at 37ºC for one hour. Once this initial digest was complete the vitreous humor and some of the neural tissue was removed by gentle agitation, dissection and abrasion. The retinas were then returned to the 3% trypsin solution for a further 15 min or until the vascular network could be easily visualised. Tissue was then transferred to a slide where it was dried, and then stained with PAS and haematoxylin, and then coverslipped using DPX as a mountant.

2.7 Immunostaining and histological staining

2.7.1 Immunocytochemistry

Both isolated cells and tissue cryosections were stained using immunocytochemical techniques. If cells were to be used, they were first washed twice in full media, whereas tissue cryosections were washed twice in PBS, until all the OCT had been removed. For tissue sections fixation was conducted using 4% PFA in PBS for 20 min at room temperature. Cells were also fixed using this method, but were alternatively fixed using 10% methanol for 5 min at -20ºC when stated. Regardless of the fixation procedure and sample type they were then washed in PBS followed by incubation in blocking solution for one hour at room temperature, consisting of 1% bovine serum albumin in PBS, with the addition of 0.2% Triton X-100 if fixed using PFA. Once the blocking step was complete samples were incubated with primary

antibody in blocking solution overnight at 4ºC. Samples were then washed twice with 1x PBS after which secondary antibody was added in blocking solution for 1 h at 37°C, followed by a further two washes with PBS. Samples were then coverslipped using Vectorshield™ as a mountant, before imaging using either a Leica SP2 AOBS confocal microscope, or a Zeiss LSM510 meta confocal system, depending upon whether an inverted or upright objective was required.

2.7.2 Haematoxylin and eosin staining

Sections cut from wax blocks were first heated to allow the wax to melt, followed by immersion in xylene for 10 min. Once complete sections were then immersed in 100% industrial methylated spirit (IMS) for 4 min, followed by a wash for 2 min in water. For sections cut from OCT blocks, slides were washed thoroughly in water to remove all remnants of OCT before continuing with the protocol. Regardless of the type of section, slides were then placed in Shandon Haematoxylin solution for a period of time consistent with the embedding process used; 12 min for wax sections, 30 s for OCT sections. Slides were then washed in water for 4 min and differentiated in 0.5% hydrochloric acid in 70% IMS for 2 min. Slides were then washed once more in water for 2 min, followed by blueing in Shandon blueing agent for 2 min, and another 2 min wash in water. Slides were then immersed in 95% IMS for 4 min before immersion in Eosin-Y for 2 min. After Eosin staining, slides were immersed in 95% IMS for 2 min, 100% IMS for 6 min and xylene for 4 min before coverslipping using DPX as mountant.

2.7.3 Periodic acid Schiff staining

Wax sections were first deparaffinised to water by treatment in xylene for 10 min, followed by 2 min incubations in decreasing concentrations of IMS: 100%, 90%, 80%, 70%, 50%, 0%(Water). Cryosections were first fixed with 4% PFA at room temperature for 20 min, followed by two washes in water before continuation of the protocol. Regardless of embedding, media sections were then oxidised in 0.5% periodic acid solution for 5 min, after which they were rinsed in distilled water and placed in Schiff reagent for up to 15 min. Sections were then washed in tap water for up to 5 min followed by counterstaining with Shandon haematoxylin solution for approximately 1 min and differentiation in 0.5% hydrochloric acid in 70% IMS for 4 min. At this point

sections were dehydrated using increasing concentrations of IMS (50%, 70%, 80%, 90%, 100%) at 4 min intervals, followed by a 4 min incubation in xylene, before coverslipping using DPX as a mountant.

2.7.4 Toluidine blue staining

Thick sections cut from resin embedded tissue as detailed above, were allowed to dry on a hot plate. Once completely dry, sections were immersed in toluidine blue solution (1% toluidine blue, 2% sodium borate) for 1-2 min at which point the slides were rinsed in tap water and coverslipped using DPX as a mountant.

2.7.5 Lead citrate staining for electron microscopy

Ultra-thin sections cut from resin embedded tissue as detailed above were stained using lead citrate and imaged on a JEOL 1010 transmission electron microscope, with images recorded using a Gatan Orius B digital camera.

2.8 Image analysis

All image analysis was conducted using Image-J software with cell widths and basement membrane thicknesses calculated using the line tool, whilst areas of mesenchymal matrix expansion were calculated using the area tool. Densitometry of western blots was also conducted using Image-J, using the integrated density tool.

Chapter 3 Results

Chapter 3 Results

Hyperglycaemia is known to affect many different proteins in endothelial cells, either by changing their levels, distribution or activity[385,287]. These changes can be detected in many ways, either by observing the behaviour of the cell, or by observing effects on the specific proteins changed by the actions of hyperglycaemia. Annexin 2 has been shown to be altered by hyperglycaemia, becoming glycated[375], and increasing its presence upon the surface of the endothelial cell [378,] where it is thought to increase the production of plasmin. As well as this extracellular role, annexin 2 has many different functions inside the cell which are relevant to diabetes and hyperglycaemia, such as the maintenance of VE-cadherin junctions[349], and intracellular trafficking[335]. To date, changes in the intracellular levels, or distribution of annexin 2 in response to hyperglycaemia have not been addressed, and therefore we set about examining the consequences of *in vitro* hyperglycaemia on annexin 2 in various endothelial cell lines.

3.1 Hyperglycaemia and expression of annexin 2

To assess the effect of hyperglycaemia on annexin 2 levels in the endothelium, hCMEC/D3 cells (human immortalised brain microvascular endothelial cells) were cultured for 1 week in various levels of glucose and mannitol. These consisted of euglycaemic 5mM glucose, 15mM glucose to represent an intermediate level of hyperglycaemia, 25mM glucose to represent a high level of hyperglycaemia, and 5mM glucose with 20mM mannitol '+20mM mannitol' to act as an osmotic control for the increased sugar load. These conditions of culture will henceforth be referred to as 'the hyperglycaemic culture conditions' to minimise repetition.

It has been reported previously that endothelial cells exposed to hyperglycaemia shuttle their annexin 2 to the surface of the cell, such that it is exposed to the lumen of the vessel[378]. To determine whether this was true in our system we exploited the calcium dependent phospholipid binding properties of the annexins and collected annexin 2 from the surface of the endothelium using the calcium chelator EGTA (ethylene glycol tetraacetic acid) to release any annexins bound. Intracellular annexin 2 was collected via cell lysis post elution of annexin 2 from the surface, and hence both 'surface' and 'intracellular' fractions of annexin 2 were assessed for changes relative to one another.

EGTA eluted fractions of annexin 2 from the surface of the endothelial cells cultured in the hyperglycaemic culture conditions were positive for both annexin 2 and tubulin when analysed via western blot (Figure 3.1). Since these samples are representative of the surface of endothelial cells they should be negative for tubulin, however, samples were consistently positive for tubulin, suggesting whole cell contamination of the sample. Although this experiment was repeated several times, eluates consistently contained tubulin, and hence this approach was abandoned.

Whilst the surface fraction of endothelial cells was difficult to assess, annexin 2 isolated from the intracellular compartment of hCMEC/D3 cells was still available for analysis. Both in the representative western blot, and the subsequent densitometry (n=3) the levels of annexin 2 did not vary significantly, suggesting that culture in varying levels of hyperglycaemia for 1 week is not sufficient to alter annexin 2 expression levels in these cells (Figure 3.2A).

This experiment was also repeated for a macrovascular cell line, namely HUVEC (human umbilical vein endothelial cells). Similar data to those gained from the hCMEC/D3 cells are shown in a representative western blot, with annexin 2 and tubulin staining and a graphical representation of the data from three experiments shown on the right (Figure 3.2B). These data also show that annexin 2 is unchanged by culture in the varying glucose culture conditions for 1 week, demonstrating that neither microvascular nor macrovascular endothelial cells change their levels of intracellular annexin 2 in response to 1 week culture in hyperglycaemia.

To assess the effect of longer term hyperglycaemia on intracellular annexin 2 in endothelial cells a third cell line, derived from immortalised rat brain endothelium (GPNT)[386] was cultured for 9 weeks in the varying glucose conditions. Due to differences in the culture medium, in this experiment the euglycaemic level was 6.1mM and the mannitol control 18.9mM, to bring the total sugar load to 25mM. Consistent with the previous two experiments no changes in annexin 2 levels were detectable once the annexin 2 protein bands were normalised to those of the tubulin loading controls (Figure 3.3). Whilst it might be possible at some time points to identify differences in the expression of annexin 2, no consistent discernable pattern was distinguished, hence the data suggest that there were no significant changes in annexin 2 expression in response to hyperglycaemia.

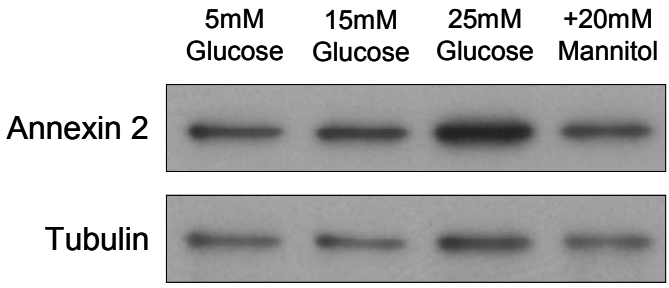

Figure 3.1: Annexin 2 isolated from the extracellular compartment is contaminated with tubulin.

Representative western blot of surface annexin 2 from hCMEC/D3 cells cultured for 7 days in the various conditions of glycaemia as shown, with a +20mM Mannitol control. Extracellular annexin 2 was then obtained using EGTA, as detailed in the materials and methods. Samples were then western blotted for annexin 2 and tubulin, also as detailed in the materials and methods, using the following antibodies: Anti-annexin 2 (1:1000; BD Biosciences), Anti-Tubulin (Clone 1A2, courtesy of Prof K.Matter), with the secondary in both cases Anti-mouse-HRP (1:5000; Dako). Protein bands were visualised using ECL

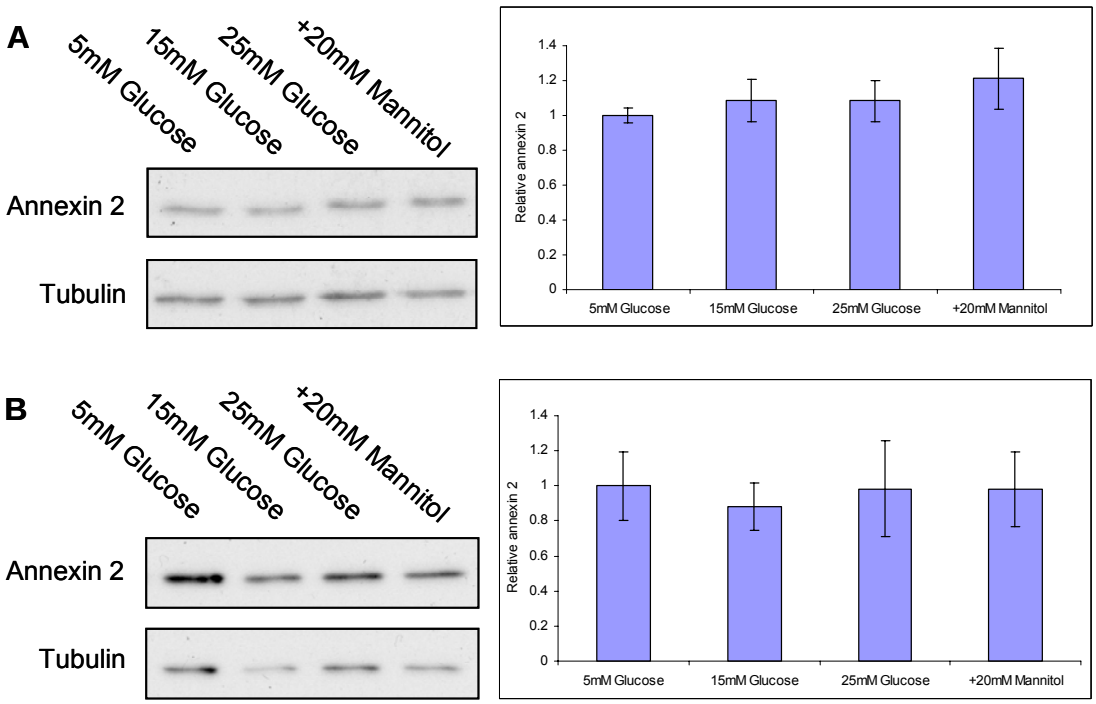

Figure 3.2: Intracellular annexin 2 protein levels do not change in response to hyperglycaemia in hCMEC/D3 or HUVEC cells.

Representative western blots from three experiments showing intracellular annexin 2 and tubulin from both hCMEC/D3 cells (A) and HUVEC (B), cultured for 7 days in the various conditions of glycaemia as shown. Cell lysis, to collect intracellular annexin 2 only, was conducted via the reducing sample buffer based protocol as detailed in the materials and methods. Samples were then western blotted for annexin 2 and tubulin, also as detailed in the materials and methods, using the following antibodies: Anti-annexin 2 (1:1000; BD Biosciences), Anti-Tubulin (Clone 1A2, courtesy of Prof K.Matter), with the secondary in both cases Anti-mouse-HRP (1:5000; Dako). Protein bands were visualised using ECL, and densitometry conducted using "Image-J" to produce graphs opposite, plotted as mean annexin 2 relative to tubulin ± s.d (n=3).

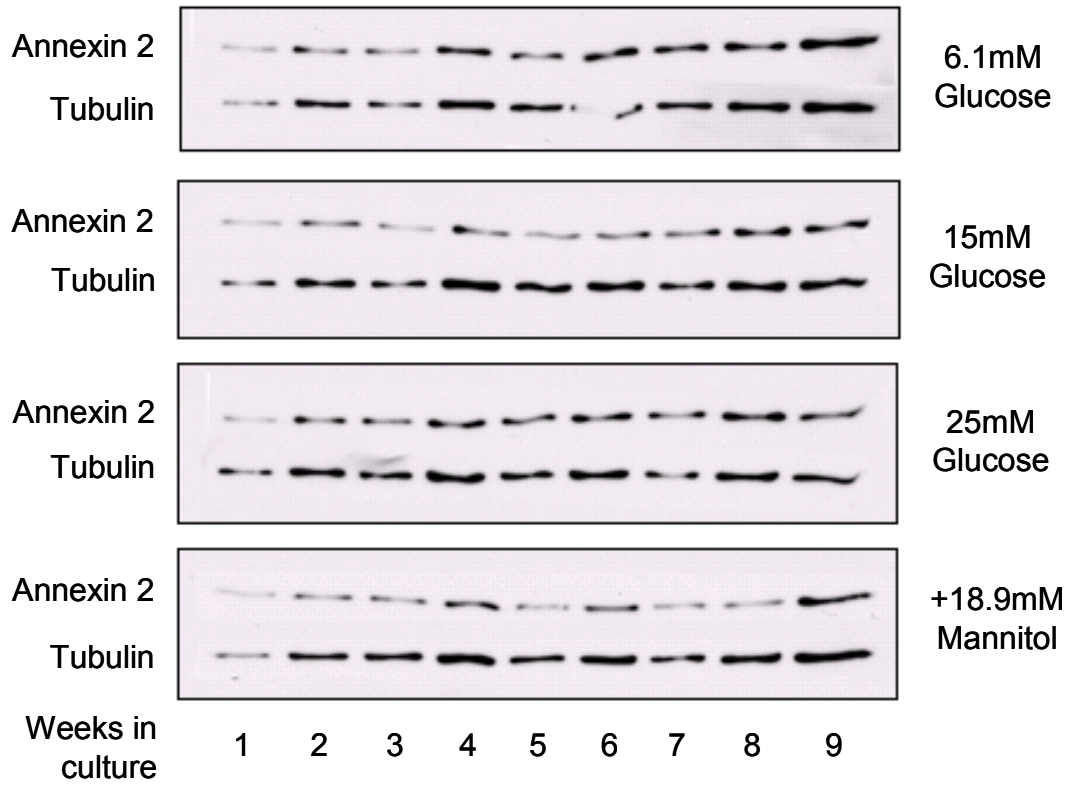

Figure 3.3: Intracellular annexin 2 in GPNT cells in a model of chronic hyperglycaemia. Changes in annexin 2 and tubulin levels in GPNT cells cultured for up to 9 weeks in the various conditions of glycaemia as shown. Cell lysis, to collect intracellular annexin 2 only was conducted via the reducing sample buffer based protocol as detailed in the materials and methods. Samples were then western blotted for annexin 2 and tubulin, also as detailed in the materials and methods, using the following antibodies: Anti-annexin 2 (1:1000; BD Biosciences), Anti-Tubulin (Clone 1A2, courtesy of Prof K.Matter), with the secondary in both cases Anti-mouse-HRP (1:5000; Dako). Protein bands were visualised using ECL.

3.2 Distributional changes of endothelial cell proteins in response to hyperglycaemia

Since various proteins in endothelial cells are known to change their distribution, level or activity in response to hyperglycaemia, we next investigated the subcellular localisation of certain proteins known to be altered in response to hyperglycamia.

GLUT-1 is one of the major glucose transporter of endothelial cells[387] and changes in its distribution/localisation as a consequence of diabetes are still under some debate[388,389]. Examination of the distribution of GLUT-1 in HUVEC grown in the varying conditions of hyperglycaemia shows that minor changes were apparent after 1 week in culture (Figure 3.4). Focusing on the staining in the perinuclear region, in comparison to those cultured in 5mM glucose, HUVEC cultured in 25mM glucose exhibited a very mild increase in the size of the region enriched for GLUT-1. In contrast, HUVEC cultured in 15mM glucose showed a reduction of signal, indicating possible differential responses to varying levels of hyperglycaemia. Since cells cultured in increased mannitol did not show any of these changes they presumably arise as a consequence of hyperglycaemia and not increased osmotic stress.

Proteins involved in the maintenance of endothelial junctions are known to alter their distribution as a consequence of diabetes. ZO-1 is a marker of tight junctions, and in HUVECs grown in hyperglycaemic conditions it can be seen that after one week of culture the distribution of ZO-1 is unaffected (Figure 3.5), with the protein enriched at the cell junctions. VE-cadherin was also examined, as an adherens junction marker specific to endothelial cells (Figure 3.6). Similar to the results obtained for ZO-1, there was no significant change in VE-cadherin distribution in HUVECs exposed to hyperglycaemia for 1 week, with continuous staining seen at the cell junctions in all culture conditions.

In these experiments cells were additionally stained for F-actin using fluorescently labelled phalloidin. In all the figures, the distribution or pattern of actin staining underwent minor changes when cells were exposed to hyperglycaemia. In comparison to cells cultured in 5mM glucose, cells cultured in hyperglycaemia displayed more stress fibers and diffuse staining around the cortex, with more distinctive changes detectable in cells cultured in 25mM glucose than 15mM glucose, suggesting a possible effect of hyperglycaemic dose. Some of these changes in F-actin

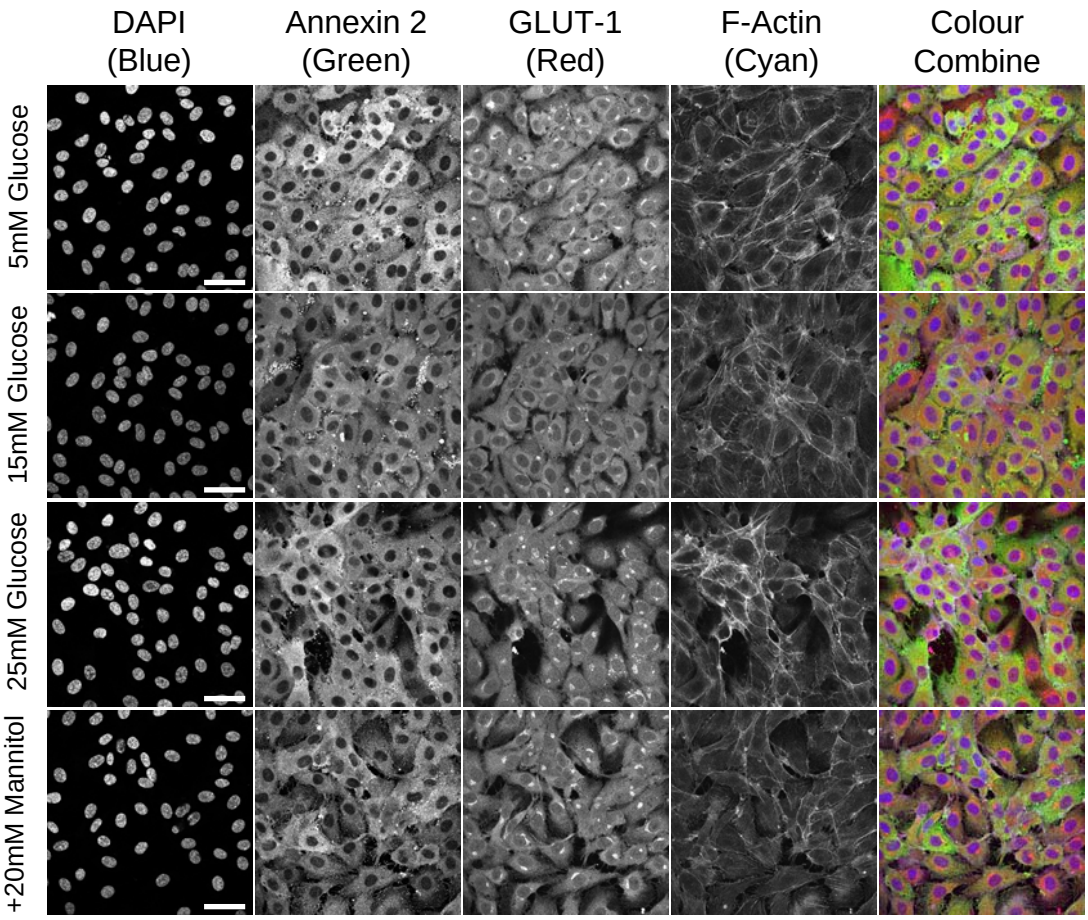

Figure 3.4: Distribution of annexin 2 and GLUT-1 in HUVEC maintained in varying glucose / mannitol concentrations for 1 week.
HUVEC were plated onto glass coverslips and allowed to grow in the various conditions of glycaemia as shown. After 1 week cells were fixed and stained as described in the materials and methods using the following antibodies and reagents; Primary antibodies, Mouse-Anti-annexin 2 'HH7' (1:100), Rabbit-Anti-GLUT-1 (1:100); Secondary Antibodies / Reagents, Anti-Mouse Alexafluor-488 (1:1000), Anti-Rabbit Alexafluor-568 (1:1000), DAPI (1:500) and Phalloidin-633 (1:60). Scale bar 50μm

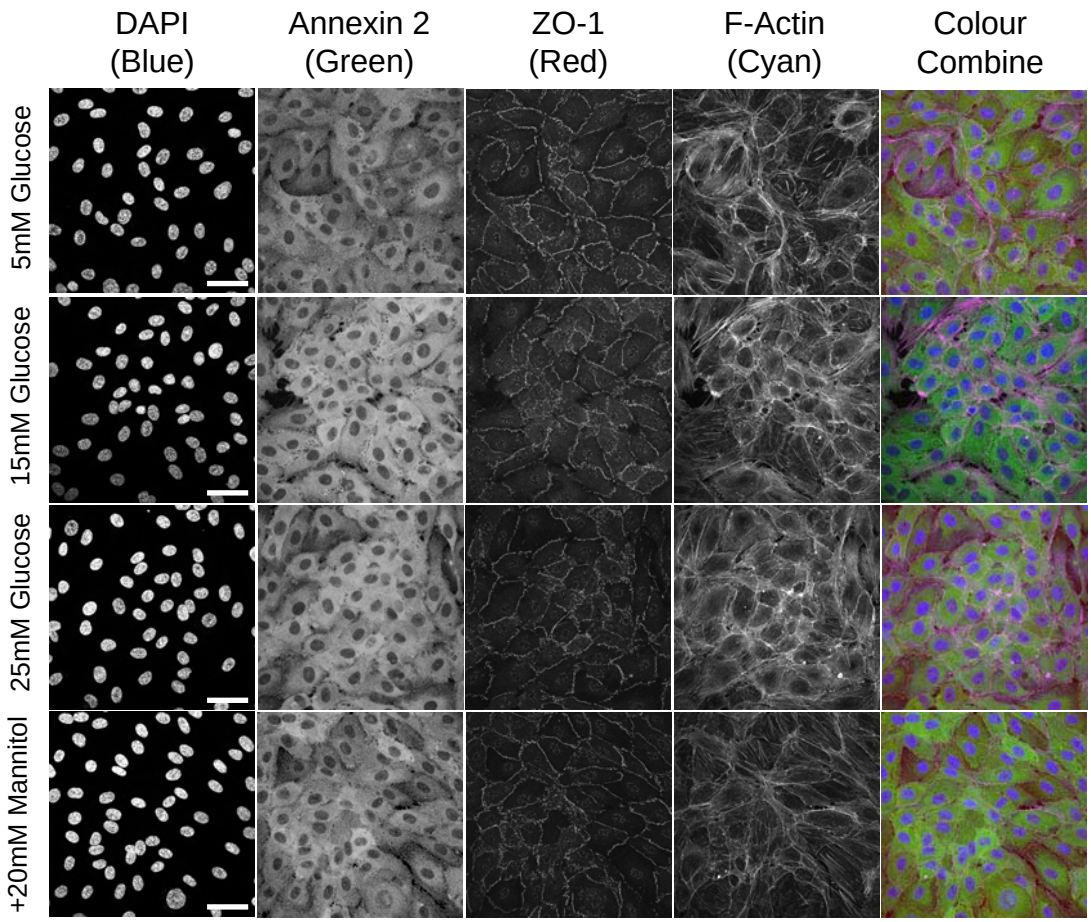

Figure 3.5: Distribution of annexin 2 and ZO-1 in HUVEC maintained in varying glucose / mannitol concentrations for 1 week.

HUVEC were plated onto glass coverslips and allowed to grow in the various conditions of glycaemia as shown. After 1 week cells were fixed and stained as described in the materials and methods using the following antibodies and reagents; Primary antibodies, Mouse-Anti-annexin 2 'HH7' (1:100), Rabbit-Anti-ZO-1 (1:300); Secondary Antibodies / Reagents, Anti-Mouse Alexafluor-488 (1:1000), Anti-Rabbit Alexafluor-568 (1:1000), DAPI (1:500) and Phalloidin-633 (1:60). Scale bar 50μm

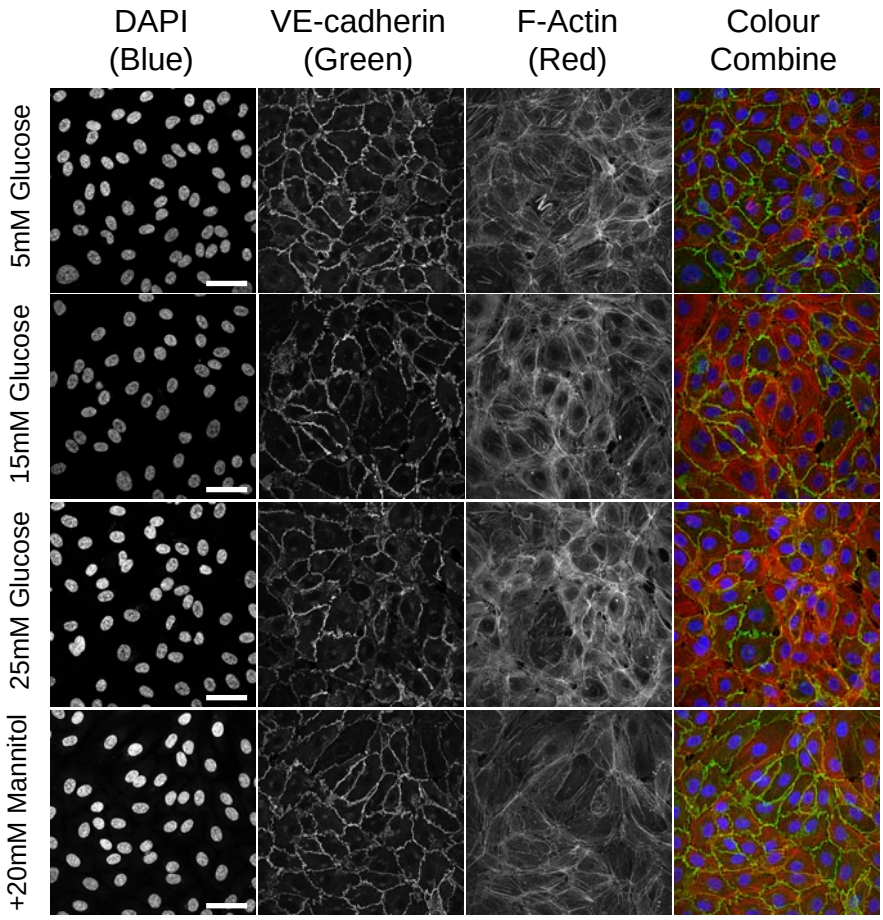

Figure 3.6: Distribution of VE-cadherin in HUVEC cells maintained in varying glucose / mannitol concentrations for 1 week.

HUVEC were plated onto glass coverslips and allowed to grow in the various conditions of glycaemia as shown. After 1 week cells were fixed and stained as described in the materials and methods using the antibodies and reagents; Primary antibodies, Mouse-Anti-VE-cadherin (1:50); Secondary Antibodies / Reagents, Anti-Mouse Alexafluor-488 (1:1000), DAPI (1:500) and Phalloidin-633 (1:60). Scale bar 50μm

distribution, can also however be detected in the cells exposed to additional mannitol, suggesting that changes may occur in response to osmotic stress.

In both figures 3.4 and 3.5 cells were also stained for annexin 2 to examine possible changes in its distribution. In both cases the distribution of annexin 2 was observed to not change in response to hyperglycaemia when examined by confocal microscopy. There was no enrichment to membranes or cellular compartments that could be detected by this method and annexin 2 staining was absent from the nucleus with uniform diffuse staining across the cytoplasm.

Annexin 2 staining was also examined in microvascular hCMEC/D3 cells to assess whether changes in localisation in response to hyperglycaemia could be observed that were not apparent in the macrovascular HUVECs (Figure 3.7A). Similar to the results obtained in HUVEC, the localisation of annexin 2 did not change in hyperglycaemic culture, with it being absent from the nuclei, and evenly distributed throughout the cell, with no localisation to any defined structures or organelles. To ensure no changes in annexin 2 staining were being masked by the selected fixation procedure, cells treated identically were subjected to methanol fixation and then stained for annexin 2 (Figure 3.7B). The staining obtained was simlar to that of PFA fixation with annexin 2 distributed throughout the cell, and no changes in localisation due to hyperglycaemia observed.

3.3 Sucrose density gradient analysis of annexin 2 in endothelial cells cultured in hyperglycaemia

Although the localisation of annexin 2 was apparently unaffected by culture in hyperglycaemia when examined by confocal microscopy, annexin 2 has been reported to move in response to hyperglycaemia, as mentioned previously, to the surface of the endothelial cell[378]. Therefore some relocalisation may occur that cannot be detected by confocal microscopy. To address this point we employed a biochemical examination of annexin 2 via sub-cellular fractionation. Three different sub-cellular fractionation protocols were used to ascertain which would be best to optimise and conduct in triplicate. Initially cells were examined using a detergent solubility fractionation method. In these experiments GPNT cells were fractionated into ‘surface’, ‘detergent soluble’ and ‘detergent insoluble’ fractions, and then examined for annexin 2 via western blotting. Annexin 2 was identified in both the surface and detergent-soluble fractions of the cell, whilst none was detectable in the detergent-

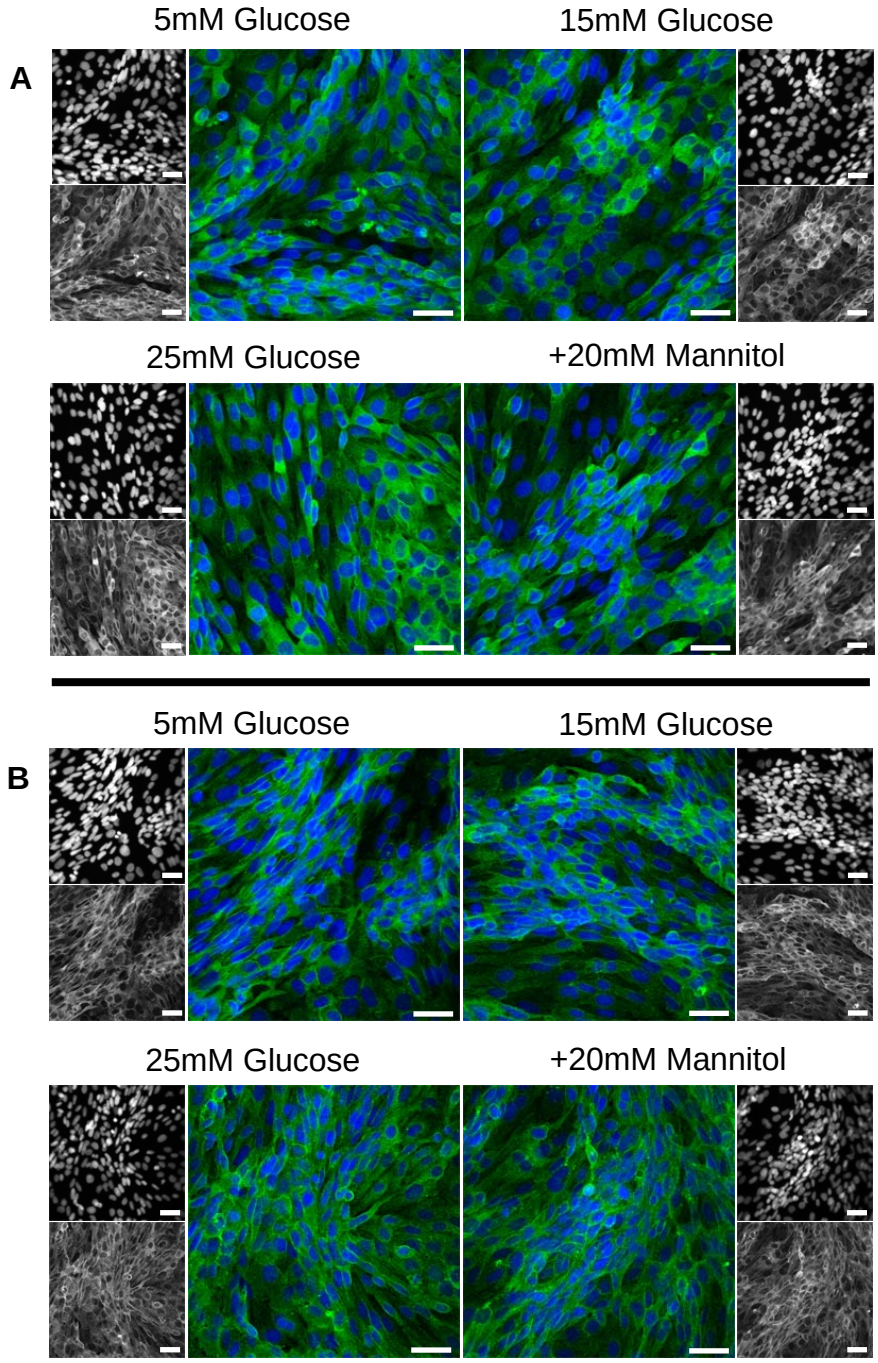

Figure 3.7: Effect of hyperglycaemia on annexin 2 in microvascular hCMEC/D3 cells.
hCMEC/D3 cells were cultured for 1 week in the varying glucose and mannitol conditions stated for 1 week, followed by fixation in 4% PFA (A) or methanol (B) as stated in the materials and methods section. Cells were then stained for DNA (blue) and annexin 2 (green) using the following antibodies and reagents: DAPI (1:500), Annexin 2 (HH7: 1:100), secondary (Anti-Mouse Alexafluor-488 1:1000). Main panel is merged image with constituents in minor panels, DAPI (upper) and annexin 2 (lower). Scale bars 50µm

insoluble fraction for all three replicates (Figure 3.8). Since no annexin 2 was detected in the detergent-insoluble fraction, and the protocol was complicated by technical issues, this method was abandoned. Using hCMEC/D3 cells Percoll™ fractionation was subsequently tested, separating annexin 2 into 'surface' and six intracellular fractions on the basis of density. Western blotting revealed annexin 2 in the surface compartment of the cell, and in four of the six intracellular fractions, peaking in fraction two (Figure 3.9). Although this method produced good fractionation it was found that for the higher density fractions the Percoll™ could not be removed, even after centrifugation for extended periods of time at 200,000 x g. This resulted in Percoll™ contamination of the higher density fractions and subsequent dilution of the protein sample, distorting the results. Hence this protocol was also discarded and we tested sucrose density centrifugation. For this experiment hCMEC/D3 cells were cultured in the varying hyperglycaemic culture conditions for 1 week and subsequently subjected to the sucrose gradient fractionation protocol (Figure 3.10). Western blotting for annexin 2 from the 12 fractions revealed staining for annexin 2 in fractions 2-8, with occasional localisation to fraction 12, forming a bell shaped curve upon quantification by densitometry. Transferrin receptor was used as a marker of early endosomes to indicate that the gradient was reproducible and that all four experiments produced a similar pattern of fractionation. Transferrin receptor was detected in fractions 5-7 regardless of glucose or mannitol load. Examining the results from the western blot for annexin 2 more thoroughly it can be seen that when hCMEC/D3 cells are cultured in 15mM and 25mM glucose there is a shift of the peak of annexin 2 to fractions of a higher density, such that in cells exposed to 15mM glucose the peak of annexin 2 shifted one fraction, whilst in cells cultured in 25mM glucose annexin 2 shifted two fractions. Furthermore, cells cultured in 5mM glucose +20mM mannitol did not exhibit this shift, indicating this to be a consequence of hyperglycaemic culture and not of osmotic stress.

Since the sucrose gradient fractionation protocol produced the most interpretable results the experiment was repeated twice more with the results amalgamated (Figure 3.10; Graph). After normalisation of the data a distinctive shift was evident for annexin 2 isolated from cells cultured in 25mM glucose, with a 'shoulder' of annexin 2 moving to denser fractions. Analysis of this graphical representation reveals a dose sensitive shift in annexin 2 to higher density fractions when cells are cultured in 15mM and 25mM glucose. This effect on annexin 2 was not a function of increased osmotic stress since cells cultured in '+20mM mannitol' exhibited a similar distribution of annexin 2

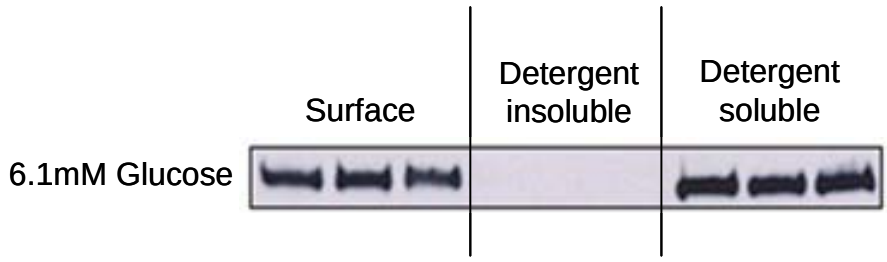

Figure 3.8: Sub-cellular fractionation of GPNT cells using the detergent based method.
GPNT cells were grown in euglycemic 6.1mM glucose for 1 week in triplicate followed by detergent based sub-cellular fractionation as described in the materials and methods. Samples were then western blotted for annexin 2, also as detailed in the materials and methods, using primary antibody anti-annexin 2 (1:1000; BD Biosciences) and secondary antibody Anti-Mouse-HRP (1:5000; Dako). Bands were visualised using ECL.

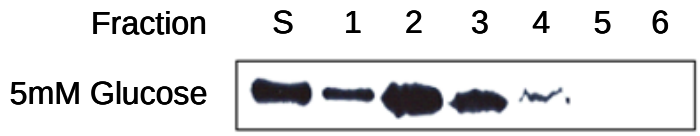

Figure 3.9: Sub-cellular fractionation of hCMEC/D3 cells using the Percoll™ based method. hCMEC/D3 cells grown in 5mM glucose for one week were subjected to the Percoll™ based sub-cellular fraction protocol as detailed in the materials and methods. Samples were then western blotted for annexin 2, also as detailed in the materials and methods, using primary antibody anti-annexin 2 (1:1000; BD Biosciences) and secondary antibody Anti-Mouse-HRP (1:5000; Dako). Bands were resolved using ECL. Fraction numbers 1-6 are indicative of the fractions of the gradients with 1 representing the lightest and 6 the heaviest, S represents annexin 2 taken from the surface of the cells prior to the fractionation.

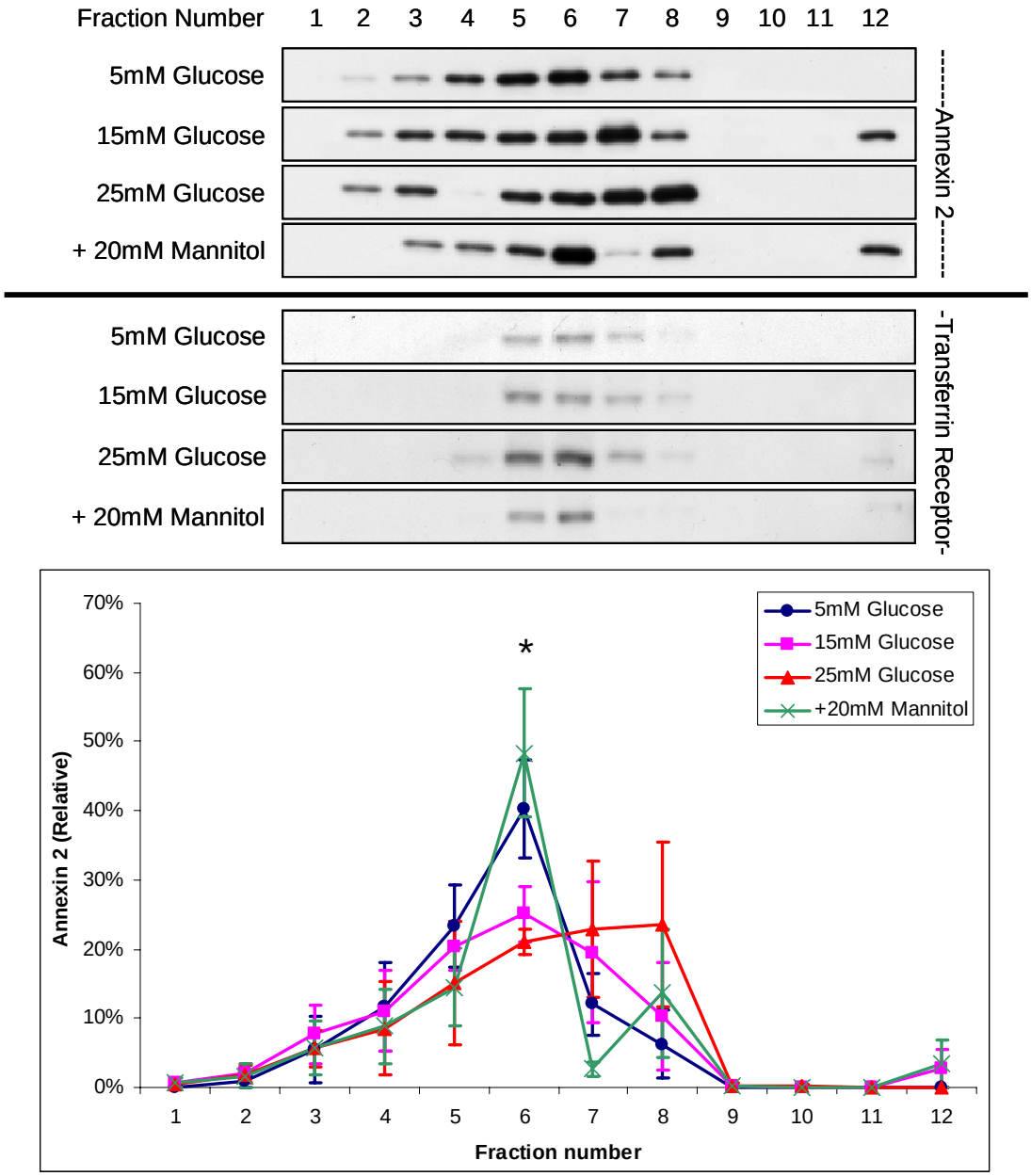

Figure 3.10: Hyperglycaemia affects the compartmentalisation of annexin 2 in hCEMC/D3 endothelial cells.

hCMEC/D3 cultured for 7 days in the various conditions of glycaemia and subjected to the sucrose gradient based method as detailed in the materials and methods. Fraction 1 is the lightest fraction whilst fraction 12 the most dense; samples were western blotted for annexin 2 and transferrin receptor, also as detailed in the materials and methods, using the following antibodies: Anti-annexin 2 (1:1000; BD Biosciences), Anti-transferrin receptor (1:1000; Invitrogen), with the secondary in both cases Anti-mouse-HRP (1:5000; Dako). Protein bands were visualised using ECL and densitometry conducted using "Image-J" to produce the graph, plotted as mean percentage annexin 2 ± sem (n=3). *=$p<0.05$ between '+20mM Mannitol' and '25mM Glucose'.

to those cultured in 5mM glucose. Statistical analysis of the normalised data using a Students t-test revealed that at fraction 6 there was a significant difference ($p<0.05$) between the levels of annexin 2 in the '+20mM mannitol' and '25mM glucose' samples, indicating a significant change in the distribution of annexin 2. These results suggest that the actions of hyperglycaemia are sufficient to move annexin 2 to a different compartment of the cell. This may occur in order for annexin 2 to fulfil a function in response to the hyperglycaemic conditions, or it could be that hyperglycaemia modifies annexin 2 in a manner that disrupts its normal cellular roles.

3.4 The localisation of various endothelial cell markers is unaffected by the absence of annexin 2

Having shown that the distribution of junctional cell markers is apparently unaffected by short term culture in hyperglycaemia, but that the subcellular compartmentalisation of annexin 2 appears to change, we next examined junctional proteins in endothelial cells lacking annexin 2. To do this brain microvascular endothelial cells from the annexin 2 knockout mouse ($AnxA2^{-/-}$) and wild-type control mice ($AnxA2^{+/+}$) were isolated and cultured. Initial examination of the endothelial cells by light microscopy revealed no obvious differences in morphology in the $AnxA2^{-/-}$ cultured cells (Figure 3.11A). To ensure that the cells isolated were endothelial in origin, VE-cadherin staining was examined via confocal microscopy in $AnxA2^{+/+}$ endothelial cells from three different areas of the cultured monolayer, demonstrating that although the morphology of the cells can vary greatly, all are indeed endothelial in origin (Figure 3.11B). Examination of VE-cadherin distribution in $AnxA2^{-/-}$ endothelial cells by this method showed continuous staining of the cell-cell junctions, equivalent to that of $AnxA2^{+/+}$ endothelial cells, demonstrating that the loss of annexin 2 does not influence the localisation of VE-cadherin in these cells (Figure 3.11C). Western blot analysis of cell lysates confirmed the absence of annexin 2 protein (Figure 3.11D). With this initial characterisation concluded we next examined of the effects of hyperglycaemia on $AnxA2^{-/-}$ endothelial cells. To achieve this $AnxA2^{-/-}$ and $AnxA2^{+/+}$ endothelial cells were cultured in the various hyperglycaemic culture conditions for one week and the localisations of VE-cadherin, ZO-1 and F-actin examined by confocal microscopy (Figure 3.12). VE-cadherin localisation was unaffected by culture in hyperglycaemia in both $AnxA2^{+/+}$ and $AnxA2^{-/-}$ endothelial cells, maintaining its localisation at the periphery of the cell. ZO-1 localisation was also consistent in the

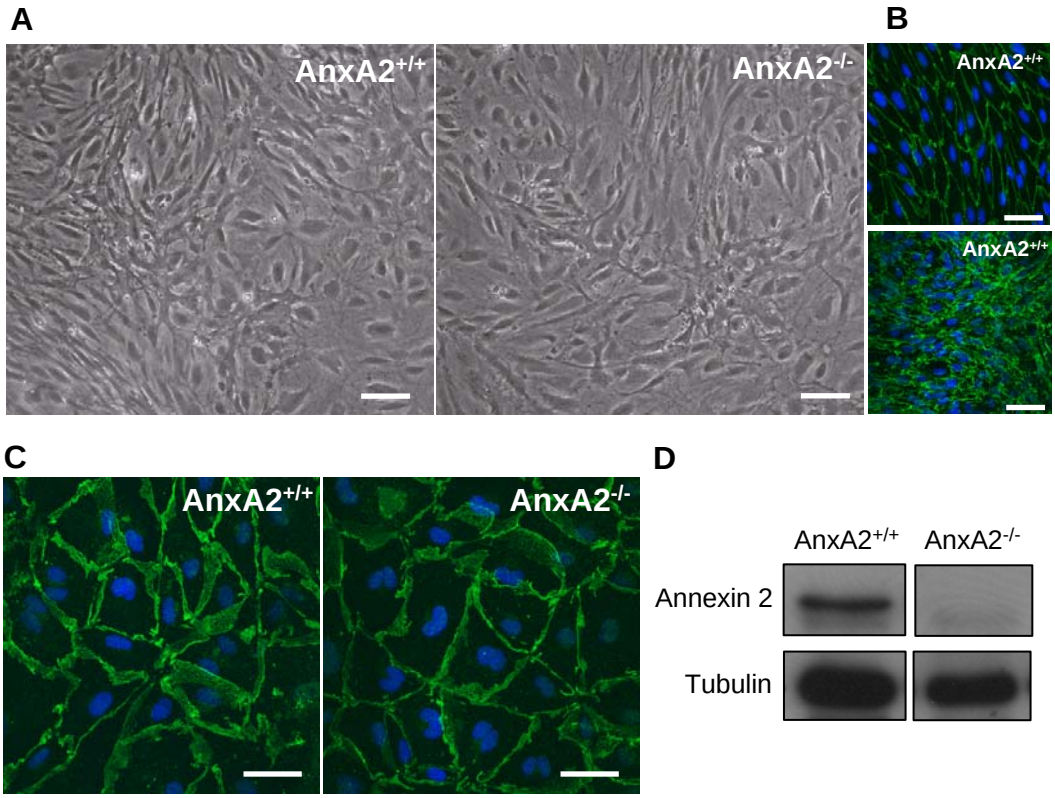

Figure 3.11: Microscopic examination of endothelial cells from AnxA2$^{+/+}$ and AnxA2$^{-/-}$ mice. AnxA2$^{+/+}$ and AnxA2$^{-/-}$ endothelial cells were isolated and cultured to confluence as detailed in the materials and methods section. Cells were imaged by light (A) and confocal (B) microscopy, to demonstrate they were endothelial in origin, with further confocal analysis demonstrating that AnxA2$^{-/-}$ endothelial cells maintain normal VE-cadherin localisation (C). Cells were then lysed using the reducing sample buffer based method, and western blotted for annexin 2 and tubulin to confirm the genotype of AnxA2$^{-/-}$ samples (D). Confocal microscopy reagents: anti-VE-cadherin (1:50) Anti-Goat-Alexafluor-488 (1:1000) and DAPI (1:500). Antibodies for western blot, Anti-annexin 2 (1:1000; BD Biosciences), Anti-Tubulin (Clone 1A2, courtesy of Prof K.Matter), with the secondary in both cases Anti-mouse-HRP (1:5000; Dako). Protein bands were visualised using ECL. Scale bar 50µm

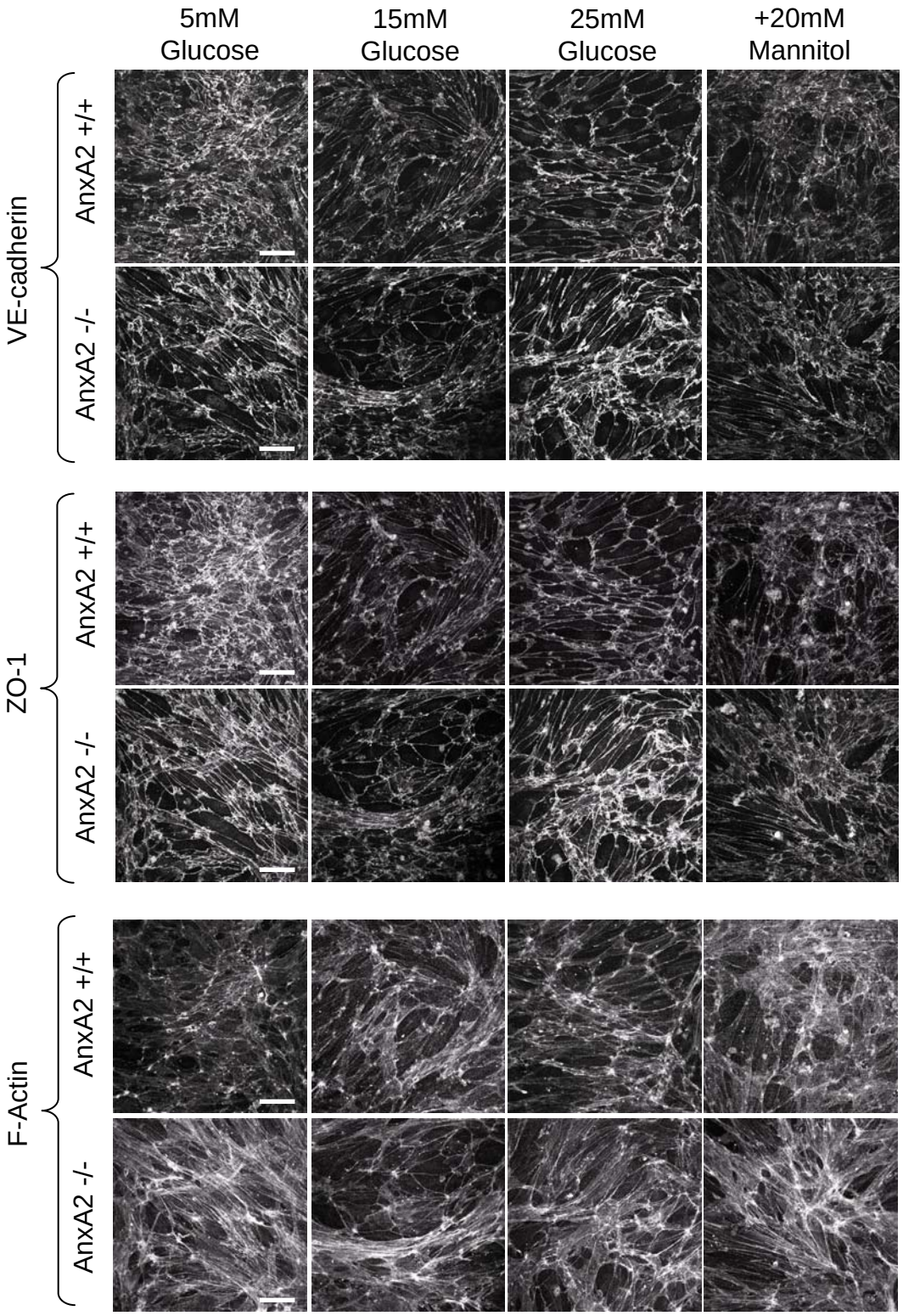

Figure 3.12: Examination of hyperglycaemia in AnxA2$^{-/-}$ endothelial cells.
AnxA2$^{+/+}$ and AnxA2$^{-/-}$ endothelial cells were isolated and cultured for one week in the various conditions of glycaemia as shown. Cells were then fixed, stained and imaged using confocal microscopy as detailed in the materials and methods. Anitbodies and reagents; Anti-VE-cadherin (1:50), Anti-ZO-1 (1:300), phalloidin-633 (1:60), Anti-mouse-Alexafluor-488 (1:1000) and Anti-rabbit-Alexafluor-568 (1:1000). Scale bar 50μm.

various culture conditions, regardless of the presence or absence of annexin 2, or culture in hyperglycaemia. Examination of F-actin in AnxA2$^{+/+}$ endothelial cells similarly demonstrated consistency in the varying culture conditions. However, in AnxA2$^{-/-}$ endothelial cells, there was a much more striking change in the localisation of F-actin, with the increased presence of actin stress fibers throughout the entire population of cells. Although striking this was not unexpected as it has previously been shown by Hayes *et al* that annexin 2 has a major role in the organisation and dynamics of the actin cytoskeleton[323]. When this increase in stress fibre formation is taken into account it appears that culturing these cells in hyperglycaemia has no discernable effects on the localisation of F-actin.

Chapter 4 Results

Chapter 4 Results

Having demonstrated subtle changes in annexin 2 in response to hyperglycaemia *in vitro*, investigations were continued in an *in vivo* setting. The streptozotocin induced model of diabetes is a well reported system described in many publications concerning diabetes research[390,391,392,393,394,395,396]. Streptozotocin specifically targets the beta-islet cells of the pancreas destroying their capability to produce insulin and hence inducing a type-1 diabetes like state. To examine the role of annexin 2 and diabetes *in vivo* we first tested the hypothesis that loss of annexin 2 would influence the severity of diabetes in this established model.

4.1 Blood glucose concentration in streptozotocin induced diabetes

One of the major effects of uncontrolled diabetes is an increase in the concentration of blood glucose. To examine this in our system we induced diabetes in wild type c57bl/6 mice using streptozotocin. In this initial study blood glucose concentrations were recorded on a fortnightly basis for the initial 8 weeks, with subsequent measurements every 4 weeks. As expected, animals with diabetes immediately exhibited a significantly higher level of blood glucose than their control littermates (Figure 4.1; blue and yellow lines) ($p<0.001$). The specific effect of loss of annexin 2 was then considered, by comparing blood glucose concentration data for annexin 2 knockout animals, with and without diabetes, hereafter referred to as $AnxA2^{-/-}$ non-diabetic and $AnxA2^{-/-}$ diabetic respectively (Figure 4.1; purple and cyan lines). The loss of annexin 2 did not significantly affect blood glucose concentration in either the non-diabetic or diabetic animals, since the degree of hyperglycaemia in the $AnxA2^{-/-}$ animals closely followed that of $AnxA2^{+/+}$.

4.2 Further examination of blood glucose concentration in streptozotocin induced diabetes

Upon analysis of the data from the first study of blood glucose levels, it was noticed that at the final time points blood glucose levels often exceeded the maximum

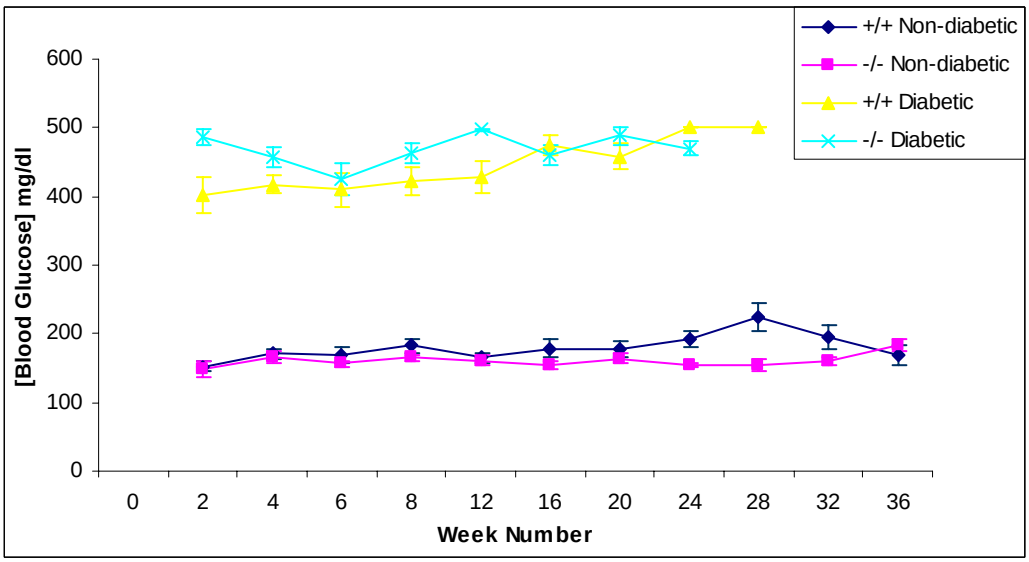

Figure 4.1: Effect of diabetes on blood glucose in AnxA2$^{+/+}$ and AnxA2$^{-/-}$ mice.
Annexin 2 wild type and null animals, both non-diabetic and diabetic, were tested for blood glucose levels at the various time points stated. Lines are mean values, from all animals available for the populations and time points, with error bars denoting ± SEM. Blue line: AnxA2$^{+/+}$ non-diabetic, purple line: AnxA2$^{-/-}$ non-diabetic, yellow line: AnxA2$^{+/+}$ diabetic and teal line: AnxA2$^{-/-}$ diabetic. n≥3

detectable for the glucose sensor at 500 mg/dl. This produced a false ceiling at this maximal value, potentially masking some of the results. To resolve this problem in subsequent studies, blood samples exceeding the recording range of the sensor were diluted in an equal volume of water and re-recorded thereby extending the potential recording range to 1000 mg/dl. There were two further issues arising from the first study that were also subsequently corrected. The first was that initially only non-fasted blood glucose levels were collected, which would hinder comparisons with current literature, since the generally accepted method is to measure fasted blood glucose levels. The second issue was that no initial starting values for blood glucose levels were obtained, thus making it impossible to accurately assess any changes in blood glucose levels following streptozotocin treatment.

With these considerations duly addressed, a new set of blood glucose levels were collected for AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic and diabetic animals. In this study animals were first fasted for 4 hours before collection of blood glucose data in order to measure fasted blood glucose levels. In addition to this, at various time points, non-fasted blood glucose concentration data were also collected (not shown). In this second study both AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic animals maintain a normal level of blood glucose, and both data sets follow each other closely. In contrast, diabetic AnxA2$^{+/+}$ and AnxA2$^{-/-}$ animals exhibited a sharp rise in blood glucose from the point of streptozotocin administration, levelling off 4 weeks later at approximately 600 mg/dl (Figure 4.2). Data from the diabetic animals were significantly different from their non-diabetic littermates at week 2 ($p<0.001$) as expected, but were broadly similar between each other with differences only apparent late in the data set at 16 weeks after onset of diabetes. Since the remaining data points were virtually identical, and because other factors commented on in the discussion may have given rise to the changes seen at week 16, the data sets from AnxA2$^{+/+}$ and AnxA2$^{-/-}$ diabetic animals were deemed to be similar.

4.3 Examination of glucose homeostasis in AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic and diabetic mice

Although comparison of AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic mice from both studies indicated that there were no substantial differences in blood glucose levels when examined over the length of the study, subtle differences were noted at some time points suggesting that annexin 2 may have a mild modulatory effect. To investigate this

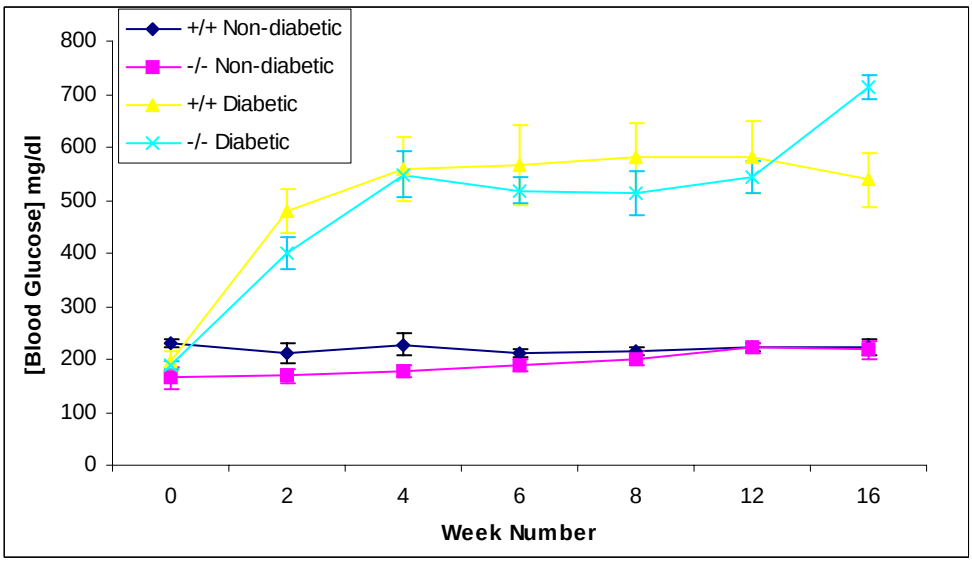

Figure 4.2: Effect of diabetes on blood glucose in fasted AnxA2$^{+/+}$ and AnxA2$^{-/-}$ mice.
Annexin 2 wild type and null animals, both non-diabetic and diabetic were fasted for 4 hours and tested for blood glucose levels at the various time points stated. Lines are mean values, from all animals available for the populations and time points, with error bars denoting ± SEM. Blue line: AnxA2$^{+/+}$ non-diabetic, purple line: AnxA2$^{-/-}$ non-diabetic, yellow line: AnxA2$^{+/+}$ diabetic and teal line: AnxA2$^{-/-}$ diabetic. n≥3

further, blood glucose concentration data were collected from all available time points both from the first and second studies. The data were then further split into non-fasted and fasted states, reflecting the change in experimental design used in the second study. Hence fasted data were only collected from the second study, whilst both the first and second studies contributed to the non-fasted data. In both fasted and non-fasted states it can be seen that the mean blood glucose levels of the non-diabetic AnxA2$^{-/-}$ animals were significantly lower than those of their AnxA2$^{+/+}$ counterparts ($p<0.001$ – non-fasted, $p<0.01$ – fasted) (Figure 4.3A). In addition, it could also be seen that upon fasting both AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic animals exhibited a significant increase in blood glucose concentration ($p<0.001$) (Figure 4.3B). Having observed that non-diabetic animals exhibit an increase in blood glucose concentration in response to fasting, data from diabetic animals were analysed in a similar way using results obtained from weeks 2-16 post streptozotocin injection. Like their non-diabetic littermates, AnxA2$^{+/+}$ diabetic animals exhibited a small increase in blood glucose concentration upon fasting from already elevated levels (Figure 4.3C), although this was not statistically significant. However, blood glucose levels from AnxA2$^{-/-}$ diabetic animals were significantly reduced in response to fasting ($p<0.001$), with the additional observation that AnxA2$^{-/-}$ animals had a significantly higher level of blood glucose when non-fasted ($p<0.001$) compared to their AnxA2$^{+/+}$ diabetic counterparts. Whether this reduction in blood glucose concentration in diabetic AnxA2$^{-/-}$ mice is a result of an abnormal response to fasting, or the presence of higher blood glucose levels when un-fasted is unclear. To further investigate and confirm this reduction in blood glucose levels in AnxA2$^{-/-}$ animals when fasted, data from those animals in the second study subjected to blood glucose concentration sampling, both before and after fasting, were plotted as a function of change in blood glucose concentration in response to fasting. These data are shown in figure 4.3D, and although they are limited by a small sample size, it is nevertheless clear that AnxA2$^{-/-}$ diabetic animals display a significant reduction in blood glucose concentration in response to fasting when compared to their AnxA2$^{+/+}$ diabetic counterparts ($p<0.01$).

4.4 Examination of weight gain in the streptozotocin model of diabetes

One of the other affects of uncontrolled type-1 diabetes is failure to gain weight (http://www.diabetes.org.uk). To examine this in our model system the weights of AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic and diabetic animals were recorded on a

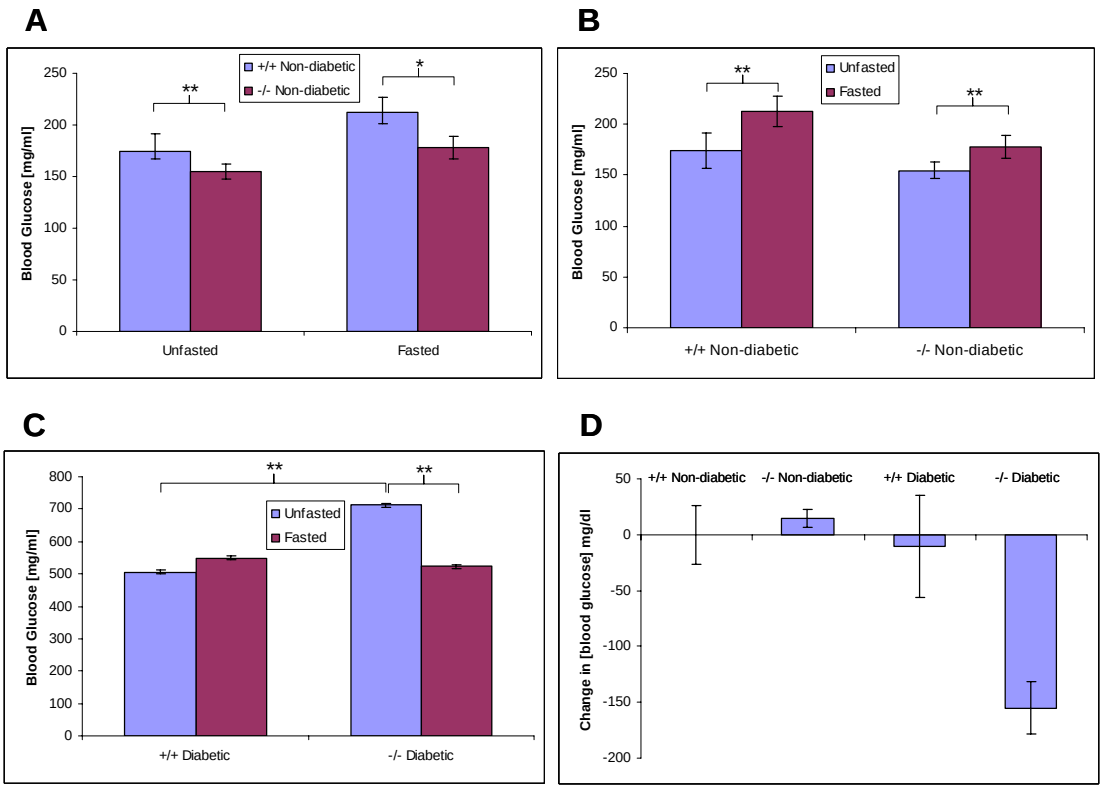

Figure 4.3: AnxA2$^{-/-}$ animals show lower basal blood glucose levels and aberrant responses to fasting.

(A,B) Blood glucose data were collected from non-diabetic animals as detailed in the materials and methods and pooled for all available time points. Data from the 1st study and unfasted data from the 2nd study were combined to create the unfasted means, with only the 2nd study contributing to the fasted mean. All data are means of all available time points, with error bars denoting ± SEM. Statistical analysis was carried out using a 2-tailed Students t-test. **(C)** Blood glucose data were pooled for weeks 2-16. Only data from the 2nd study were used to calculate the means. All data are means from the selected time points, with error bars denoting ± SEM. Statistical analysis was carried out using a 2-tailed Students t-test. **(D)** Selected animals were subjected to blood glucose tests both pre and post fasting with the differences used to create the mean values shown. Error bars denote +/- SEM and statistical analysis was carried out using a 2-tailed students t-test. *=p<0.01 **=p<0.001

fortnightly basis for the initial 8 weeks, with subsequent measurements every 4 weeks in both studies.

Over the 40 week period AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic animals gained weight in a similar manner (Figure 4.4A). Data from the AnxA2$^{+/+}$ and AnxA2$^{-/-}$ diabetic mice confirmed that streptozotocin induced diabetes is sufficient to suppress weight gain, with the difference in weights becoming significantly different to the non-diabetic counterparts at week 4 ($p<0.05$). The data also demonstrate that the loss of annexin 2 does not noticeably modulate the effect of diabetes on weight gain. Data from the second study can are shown in figure 4.4B, with non-diabetic animals gaining weight throughout the 16 week period, whereas diabetic animals failed to gain weight over that same period. The differences in weight gain were significantly different between non-diabetic and diabetic animals for both AnxA2$^{+/+}$ and AnxA2$^{-/-}$ animals at week 2 ($p<0.05$), two weeks earlier than that of the first study.

4.5 Fluorescein angiography and analysis of pericyte dropout

Having shown that AnxA2$^{-/-}$ diabetic animals have both differences and similarities to their AnxA2$^{+/+}$ counterparts it was decided to assess whether the loss of annexin 2 might influence the vascular pathology associated with diabetic retinopathy, either promoting or protecting against vascular leakage. The classical method whereby vascular changes are measured in the eye is fluorescein angiography, and therefore we set about examining our diabetic animals for signs of disease. Six mice from AnxA2$^{+/+}$ and AnxA2$^{-/-}$ control and diabetic groups, 16 weeks post streptozotocin-induced diabetes, were anaesthetised, injected with fluorescein and the resultant fluorescent retinal image recorded at 2, 4, 6, 8 and 10 minutes post-injection. Representative images from both AnxA2$^{+/+}$ and AnxA2$^{-/-}$ control and diabetic animals show that no vascular leakage was apparent at 16 weeks post diabetes (Figure 4.5A), since no radiating fluorescent signal, as illustrated in figure 4.5B, can be seen. The retinal vessels examined showed no signs of leakage in any of the animals, nor was there any evidence of microaneurysms or neovascularisation, indicating the absence of overt diabetic retinopathy in these animals at this age. The movies of the fluorescein angiograms are appended to the inside back cover of this thesis. However, it should be noted that a key aspect of diabetic retinopathy in the streptozotocin induced model of diabetes is that signs and symptoms can take well over one year to present; thus the animals examined may simply have not been old enough. However, to investigate the vasculature more closely

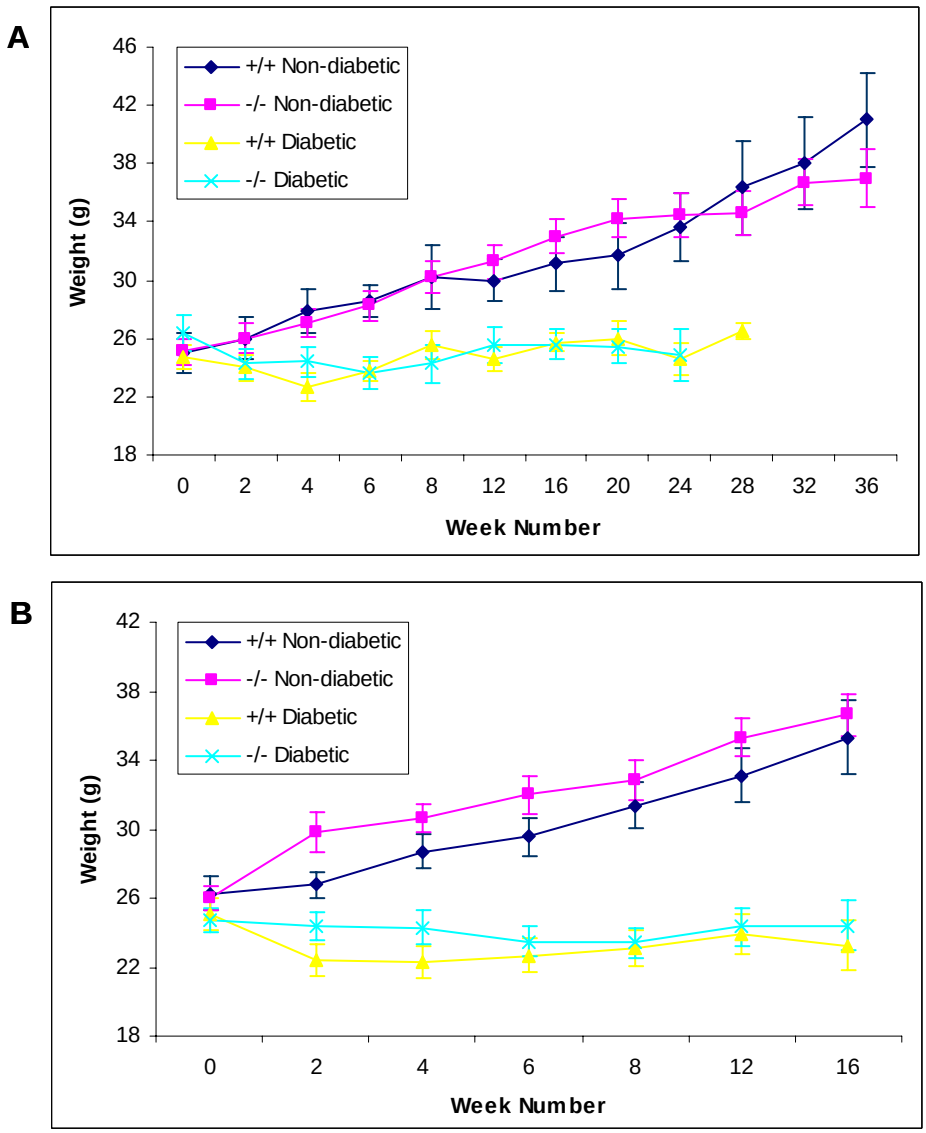

Figure 4.4: Effect of diabetes on weight gain in AnxA2$^{+/+}$ and AnxA2 $^{-/-}$ mice.
Annexin 2 wild type and null animals, both non-diabetic and diabetic, were weighed at the various time points indicated. Lines are mean values, from all animals available for the populations and time points, with error bars denoting ± SEM. Blue line: AnxA2$^{+/+}$ non-diabetic, purple line: AnxA2$^{-/-}$ non-diabetic, yellow line: AnxA2$^{+/+}$ diabetic and teal line: AnxA2$^{-/-}$ diabetic. n≥3

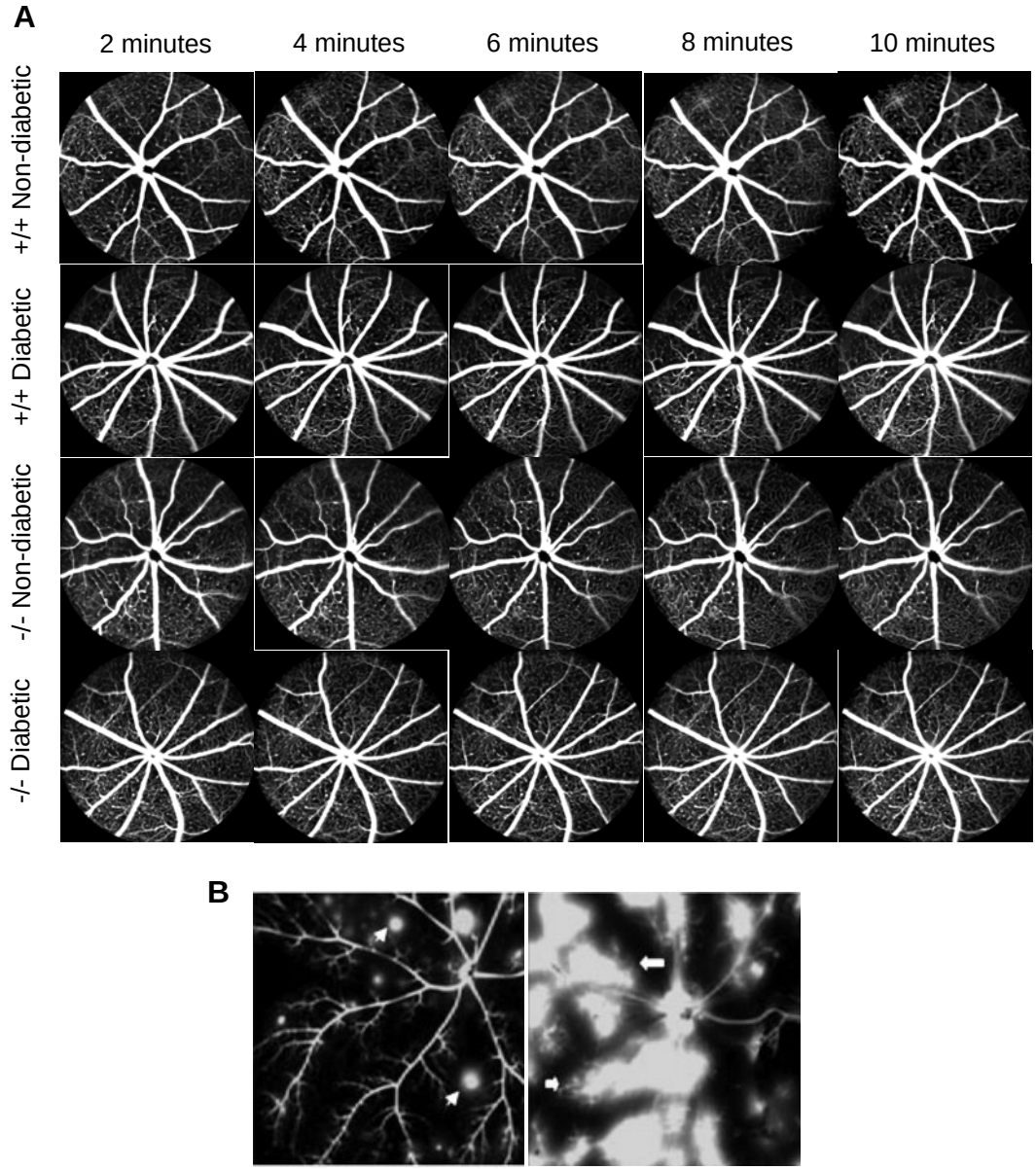

Figure 4.5: Fluorescein angiography of non-diabetic and diabetic AnxA2$^{+/+}$ and AnxA2$^{-/-}$ mice.

(A) Animals from all four groups were subjected to fluorescein angiography as described in the materials and methods. Images were taken at 2 minute time points up to 10 minutes, with the optic nerve head in the centre and vessels radiating outward. The movies from which these stills were taken is available on the DVD attached to the back cover of this thesis. (B) Image taken from Chen. Y. *et al,* (2009), Microvascular Research, **78**(1), p119-127, to illustrate the appearance of vascular leakage from retinal vessels (arrows).

and with better resolution, retinas from AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic and diabetic animals were subjected to a trypsin digest protocol and stained with periodic acid Schiff (PAS) stain, counterstained with haematoxylin. Figure 4.6 shows a representative image from the n=3 samples examined for each group, with the graphical representation of the endothelial cell: pericyte ratio below. In each image endothelial and pericyte nuclei were counted, identified by their different morphology[397,398,399,400,401], and a ratio calculated of the number of pericytes per endothelial cell. Averages from the analysis demonstrate that whilst diabetic animals have significantly fewer pericytes per endothelial cell than their non-diabetic littermates ($p<0.05$), there were no significant differences between AnxA2$^{+/+}$ and AnxA2$^{-/-}$ mice, in both the non-diabetic and diabetic states. This would indicate that whilst the action of diabetes is sufficient to reduce the number of pericytes, the loss of annexin 2 does not exacerbate or attenuate this effect.

4.6 Retinal stress in diabetic AnxA2$^{+/+}$ and AnxA2$^{-/-}$ animals

Since no microaneurysms were detected, and the pericyte to endothelial ratios in retinas from AnxA2$^{-/-}$ diabetic animals were essentially identical to those of their AnxA2$^{+/+}$ counterparts, an additional symptom of diabetic retinopathy was assessed. When the retina becomes stressed through diabetes one of the initial events to occur is the up-regulation of glial fibrillary acidic protein (GFAP) in Müller cells. GFAP is normally only expressed, and at low levels, by retinal astrocytes, but can be dramatically up-regulated in Müller cells in a stressed or diabetic retina. AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic and diabetic eyes were thus isolated from animals 16 weeks post streptozotocin or sham injection, cryosectioned, stained for GFAP protein, and examined by confocal microscopy. Retinas from both AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic animals displayed nuclei and F-actin staining indicative of normal retinal architecture, with GFAP positive cells in the ganglion cell layer (Figure 4.7, arrows). The level and area of the ganglion cell layer positive for GFAP appears a little more extensive in the non-diabetic AnxA2$^{-/-}$, but not so high as to suggest a significant glial cell activation. Diabetic animals also showed nuclei and F-actin staining indicative of normal retinal architecture but in both cases exhibited slightly higher levels of GFAP staining in the ganglion cell layer in comparison to their control littermates, suggesting modest up-regulation of GFAP protein in this region of the retina. However, since GFAP

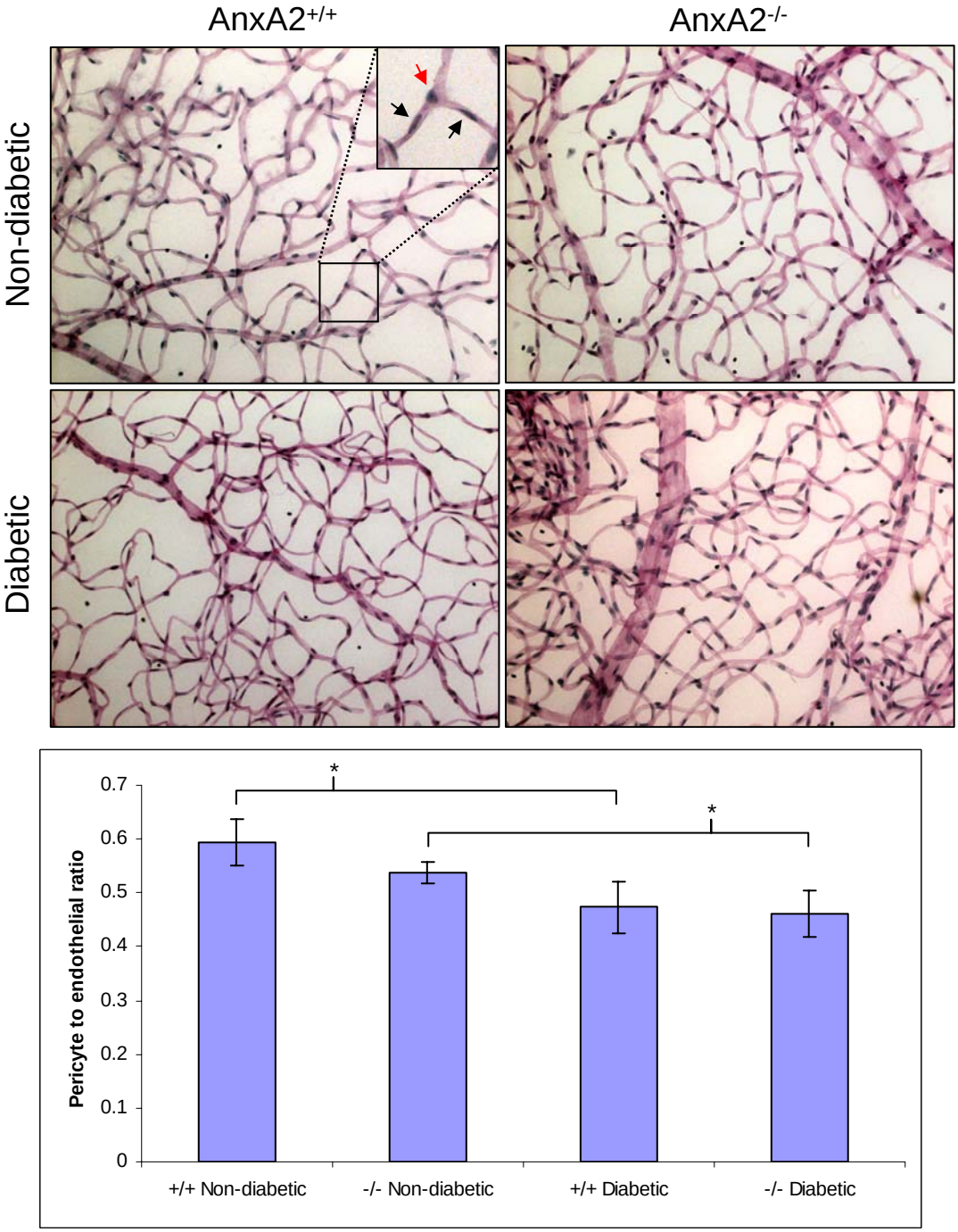

Figure 4.6: Trypsin digests of retinas from non-diabetic and diabetic AnxA2$^{+/+}$ and AnxA2$^{-/-}$ mice.

Eyes were taken from animals subjected to 16 weeks diabetes and their non-diabetic littermate controls, retinas were isolated followed by digestion with trypsin to remove the non-vascular cells. Retinal preparations were then stained with PAS and counterstained with H&E to reveal pericyte (Red arrow) and endothelial cell nuclei (Black arrows). Nuclei were then counted per field of view for n=3 samples, and pericyte to endothelial cell ratios calculated, averages ± SD of which can be seen in the graph. *=p<0.05

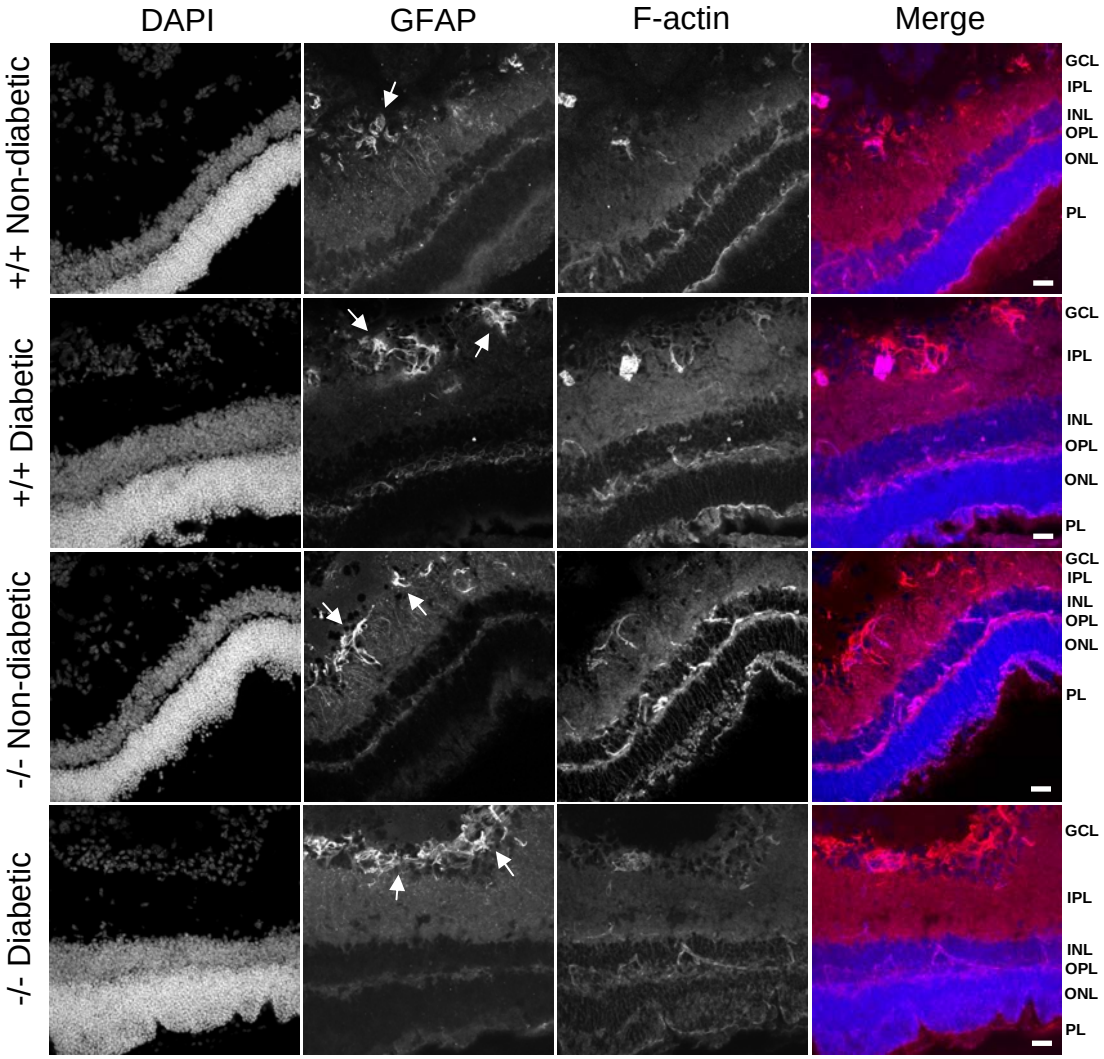

Figure 4.7: GFAP staining of retins from non-diabetic and diabetic AnxA2$^{+/+}$ and AnxA2$^{-/-}$ mice.

Eyes (n=3) from all four groups were isolated, enucleated and cryosectioned as detailed in the materials and methods. 10µM sections were fixed in 4% PFA and stained for DNA (Blue) GFAP (Red) and F-actin using Anti-GFAP (1:100), DAPI (1:500), Phalloidin-633 (1:60), Anti-Goat-Alexafluor-568 (1:1000). Arrow head indicate GFAP positive cells. Scale bar 20µm, labels of retinal layers, PL-Photoreceptor layer, ONL-Outer nuclear layer, OPL-Outer plexiform layer, INL-Inner nuclear layer, IPL-Inner plexiform layer, GCL-Ganglion cell layer.

up-regulation in Müller cells as a consequence of diabetes is usually detected in the outer plexiform layer, and none of the data showed staining in that area of the retina, it was deemed that GFAP was not significantly up regulated in these cells.

4.7 Histological examination of AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic and diabetic retinas

Having detected only modest changes of GFAP expression in the AnxA2$^{-/-}$ diabetic retina in comparison to control, although not at the level of Müller cells, other early changes that are thought to occur in the progression of diabetic retinopathy, such as the death of photoreceptors[402], were examined using histological methods. First, 10 µm cryosections taken from the same eyes examined for GFAP staining, were also stained using haematoxylin and eosin (H&E) and periodic-acid Shiff (PAS). However, the quality of the tissue was such that no reasonable interpretation of the results could be made, due to the nature of the cryogenically-embedded sections, and the samples were discarded. Due to this an improved method of sample preparation was employed, in which fresh eyes were isolated from AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic and diabetic animals 16 weeks post streptozotocin or sham injections. Eyes were then fixed in 2% glutaraldehyde / 2% paraformaldehyde, embedded in araldite resin, sectioned to 0.7 µm and stained with toluidine blue. The various layers of the retina are clearly distinguishable, allowing for more detailed interpretation than would have been possible using cryosections (Figure 4.8). Histological changes examined at this level would manifest as either changes in retinal thickness, or the appearance of anatomical anomalies. Within the different groups, the thickness of the retina varied to some extent, most likely due to irregularities in the orientation of the optic cup during sectioning. The apparent differences in total retinal thickness should therefore be deemed insignificant. Comparison of retinas between AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic and diabetic samples yielded no overt changes in the relative thicknesses of each of the individual layers of the retina. Since there were also no cellular anomalies detected, the absence of annexin 2 or presence of diabetes was judged to have no major influence on retinal histology.

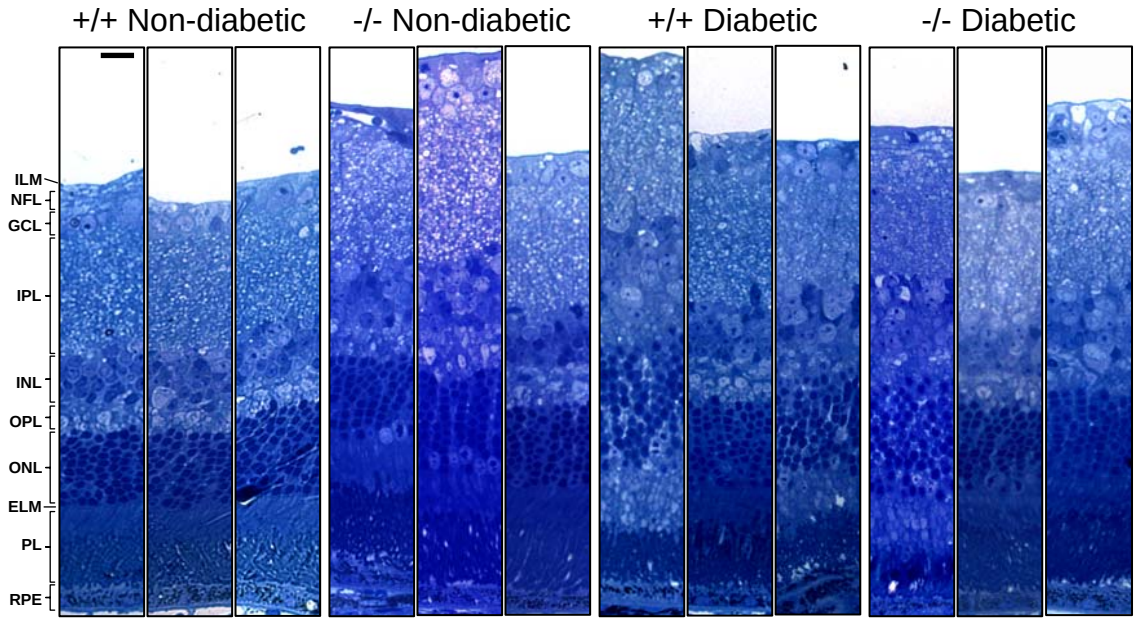

Figure 4.8: Toluidine Blue staining of retinas from AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic and diabetic animals.

Eyes from AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic and diabetic animals (n=3 each) were enucleated, fixed in 2% paraformaldhyde / 2% glutaraldehyde and embedded in araldite resin as detailed in the materials and methods. Blocks were then sectioned at 0.7µm and stained with toluidine blue, also as detailed in the materials and methods. Sections are taken from points nearest the optic nerve head, with the internal limiting membrane at the top of the image and RPE at the bottom. Scale bar 10µm, labels of retinal layers, RPE-Retinal pigment epithelium, PL-Photoreceptor layer, ELM-External limiting membrane, ONL-Outer nuclear layer, OPL-Outer plexiform layer, INL-Inner nuclear layer, IPL-Inner plexiform layer, GCL-Ganglion cell layer, NFL-Nerve fiber layer, ILM-Inner limiting membrane.

4.8 Annexin 2 and the endothelial response to VEGF

Physical changes that occur in the retina as a consequence of diabetic retinopathy may be induced by factors other than hyperglycaemia. One of the signalling molecules most strongly implicated in the progression of diabetic retinopathy is vascular endothelial growth factor (VEGF)[196,197,198]. With the physical manifestations of diabetic retinopathy examined, and no significant changes found at 16 weeks post diabetes onset, we next investigated whether the loss of annexin 2 alters, or modulates, the signalling pathways downstream of VEGF. VEGF can stimulate various responses in endothelial cells, one of which is an increase in permeability, through the re-distribution of VE-cadherin[104]. To assess whether this was true of endothelial cells lacking annexin 2 we conducted a series of studies using freshly isolated primary microvascular endothelial cells from the brains of $AnxA2^{+/+}$ and $AnxA2^{-/-}$ non-diabetic mice, and examined potential changes in junctional protein localisation in response to VEGF. The results of the first study are shown in figures 4.9-4.11. In these experiments cells were serum-starved overnight before stimulation with VEGF (50ng/ml) in fresh media. The distributions of three proteins were examined in each of the cell cultures, namely VE-cadherin (Figure 4.9), β-catenin (Figure 4.10) and F-actin (Figure 4.11). $AnxA2^{+/+}$ endothelial cells treated with vehicle alone maintained the localisation of VE-cadherin at the cell junctions with no change of signal (Figure 4.9). In contrast, $AnxA2^{+/+}$ endothelial cells treated with 50ng/ml VEGF showed a loss of VE-cadherin from the junctions at 30 minutes, still evident at 1 hour, with localisation to the junctions restored at 6 hours. Examination of the action of VEGF on VE-cadherin in $AnxA2^{-/-}$ endothelial cells yielded a contrasting result, since these cells treated with vehicle alone lost their VE-cadherin localisation at the junctions at 30 minutes, with restoration of junctional VE-cadherin at 3-6 hours, much like the $AnxA2^{+/+}$ cells treated with VEGF. $AnxA2^{-/-}$ endothelial cells treated with VEGF showed a different pattern, in that VE-cadherin localisation was disrupted at 30 minutes and 3 hours, which was more pronounced than observed in cells treated with vehicle alone at 1 hour, with junctional localisation restored by 6 hours. Localisation of the two other proteins investigated in this experiment, namely β-catenin and F-actin was also examined. β-catenin (Figure 4.10) was used as a control marker for adherens junction localisation and exhibited the distributional changes seen in VE-cadherin throughout all the different samples in this experiment. F-Actin (Figure 4.11) distribution also changed, with less cortical F-actin

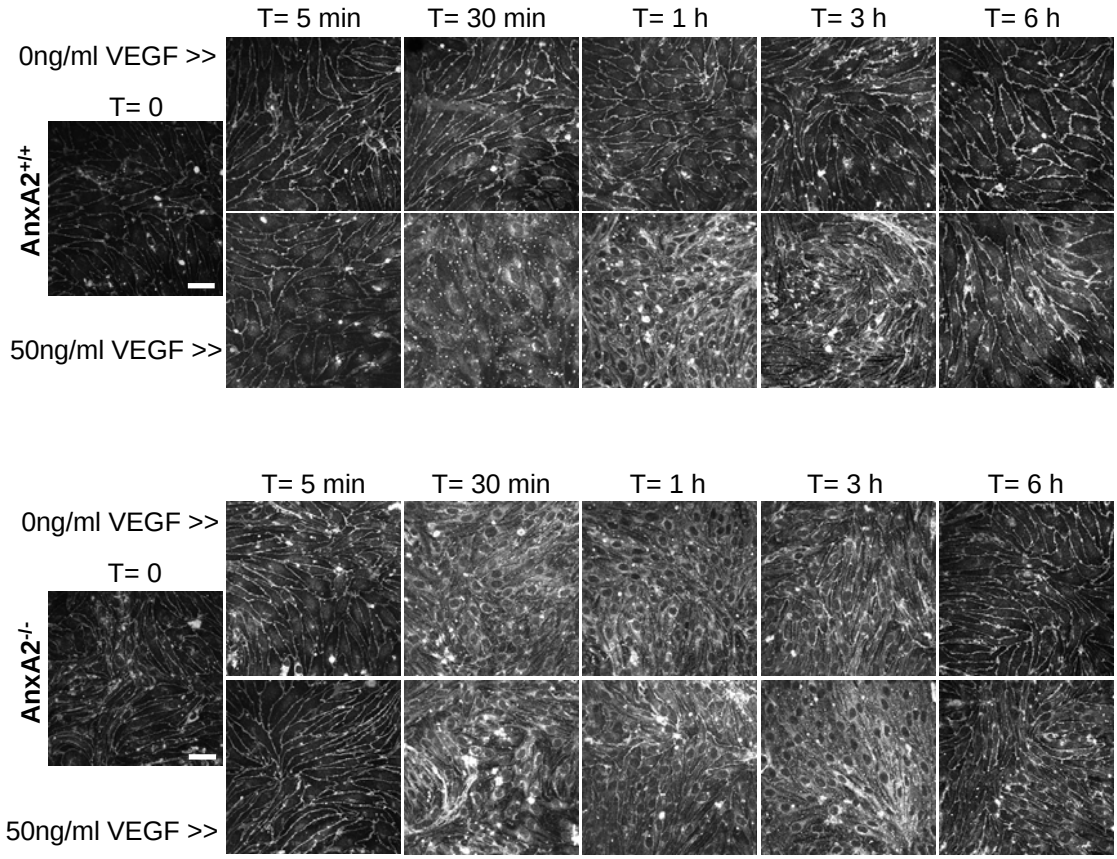

Figure 4.9: Effect of VEGF on VE-cadherin localisation in AnxA2$^{+/+}$ and AnxA2$^{-/-}$ endothelial cells.

AnxA2$^{+/+}$ and AnxA2$^{-/-}$ primary endothelial cells were isolated and exposed to 50 ng/ml VEGF or vehicle (0.1% BSA, PBS) protocol as detailed in the materials and methods. Cells were then fixed in 4% PFA at the various time points indicated and stained for VE-cadherin using Anti-VE-cadherin (1:50) and Anti-goat-Alexafluor-488 (1:1000). Scale bar 50 µm

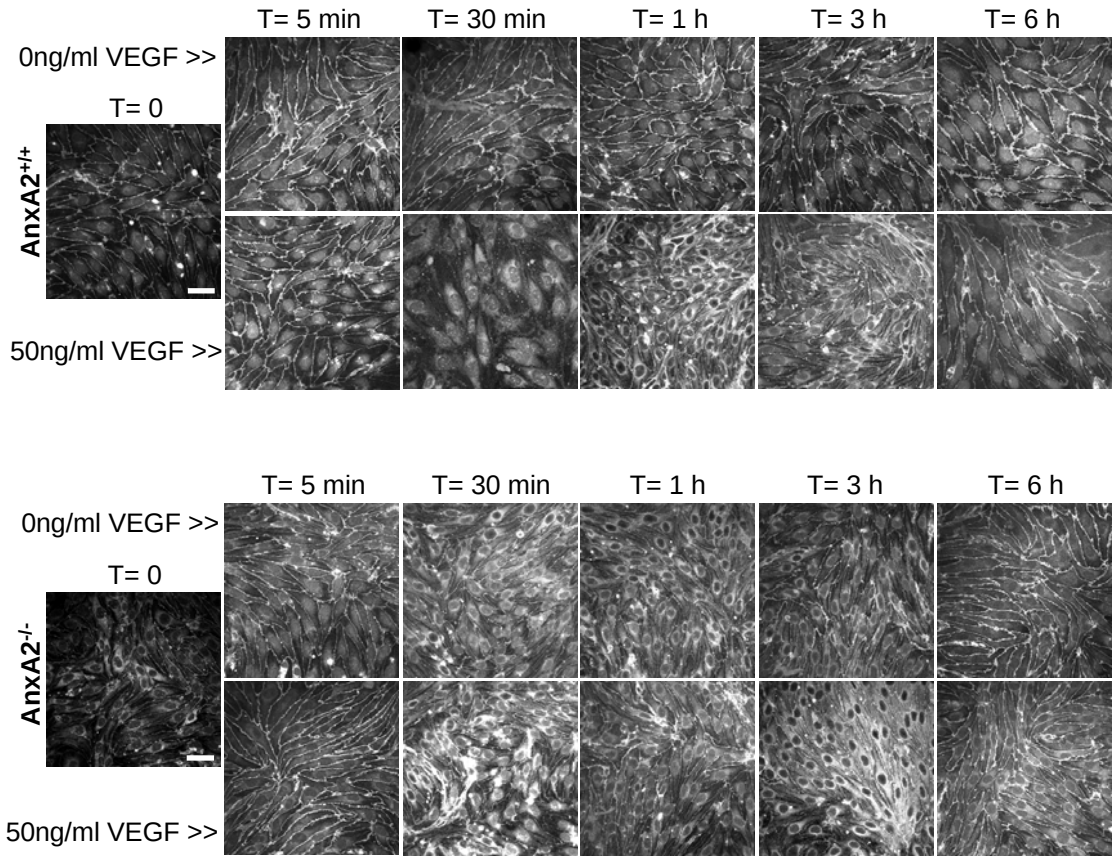

Figure 4.10: Effect of VEGF on β-catenin localisation in AnxA2$^{+/+}$ and AnxA2$^{-/-}$ endothelial cells.

AnxA2$^{+/+}$ and AnxA2$^{-/-}$ primary endothelial cells were isolated and exposed to 50 ng/ml VEGF or vehicle (0.1% BSA, PBS) protocol as detailed in the materials and methods. Cells were then fixed in 4% PFA at the various time points indicated and stained for β-catenin using Anti-β-catenin (1:100) and Anti-rabbit-Alexafluor-568 (1:1000). Scale bar 50 μm

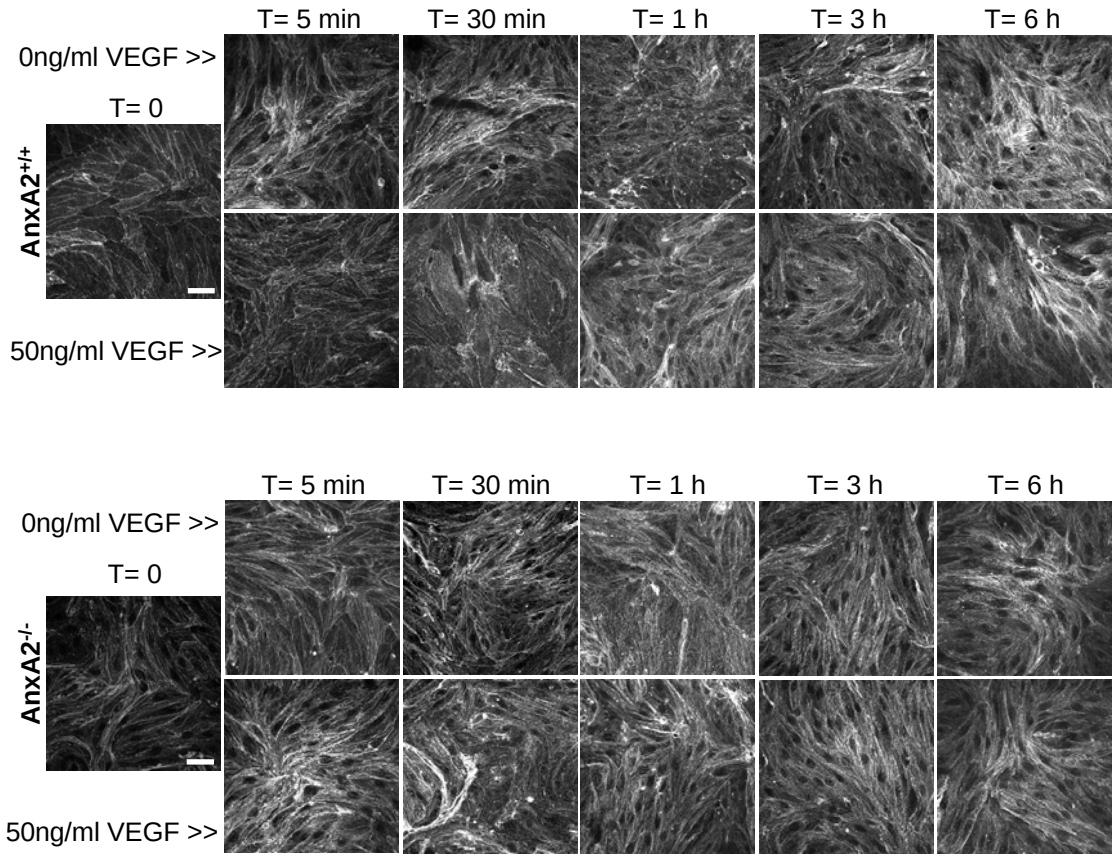

Figure 4.11: Effect of VEGF on F-actin localisation in AnxA2$^{+/+}$ and AnxA2$^{-/-}$ endothelial cells.

AnxA2$^{+/+}$ and AnxA2$^{-/-}$ primary endothelial cells were isolated and exposed to 50 ng/ml VEGF or vehicle (0.1% BSA, PBS) protocol as detailed in the materials and methods. Cells were then fixed in 4% PFA at the various time points indicated and stained for F-actin using Phalloidin-633 (1:60). Scale bar 50 μm

evident when VE-cadherin had been mobilised from the cell junctions by VEGF, returning to a normal distribution by 6 hours post VEGF or vehicle addition in all samples. Although the observations of VE-cadherin re-distribution suggest that AnxA2$^{-/-}$ endothelial cells have an alternative response to the action of VEGF, the loss of VE-cadherin from the junctions of AnxA2$^{-/-}$ endothelial cells treated with vehicle alone suggests an unusual sensitivity of junctional proteins in the AnxA2$^{-/-}$ endothelial cells to environmental changes.

With these considerations in mind, we altered the protocol to eliminate the problem of loss of junctional VE-cadherin in cells treated with vehicle alone. To do this 50% of the media left on cells overnight was removed, mixed with VEGF to yield a final concentration of 50ng/ml, and then gently added back to minimise the shock to the cells. The results are shown in Figure 4.12-4.14, and unlike the initial experiment in which AnxA2$^{+/+}$ and AnxA2$^{-/-}$ endothelial cells were treated with vehicle alone, VE-cadherin (Figure 4.12), β-catenin (Figure 4.13) and F-actin (Figure 4.14) distributions were all unchanged at all time points with vehicle alone. However, the actions of VEGF on both AnxA2$^{+/+}$ and AnxA2$^{-/-}$ cells under these conditions were also markedly reduced compared to the first experiment, with only the 5 minute time point showing any difference, and only with regard to VE-cadherin localisation. In all the experiments β-catenin was maintained at the junctions and hence can be used as a marker for junctional integrity. F-Actin structures were also constant in this experiment, indicating that the action of VEGF was not sufficient to change its distribution regardless of the presence or absence of annexin 2. However, comparison of VE-cadherin and β-catenin localisation at 5 minutes post VEGF treatment demonstrated that whilst AnxA2$^{+/+}$ cells maintained the enrichment of VE-cadherin at the junctions of the cell, AnxA2$^{-/-}$ cells did not (black arrow heads; Figure 4.15), suggesting that VE-cadherin is more readily mobilised away from junctions by VEGF in cells lacking annexin 2.

4.9 AnxA2$^{-/-}$ endothelial cells isolated from diabetic mice display disorganised VE-cadherin

Since the progression of diabetic retinopathy and the actions of VEGF are mediated in part through alterations in VE-cadherin localisation, we examined whether diabetes *in vivo* would have any effects on VE-cadherin that could be visualised in cultured endothelium. In the previous chapter endothelial cells isolated from cell lines and primary brain endothelium were exposed to hyperglycaemia for 1 week *in vitro*

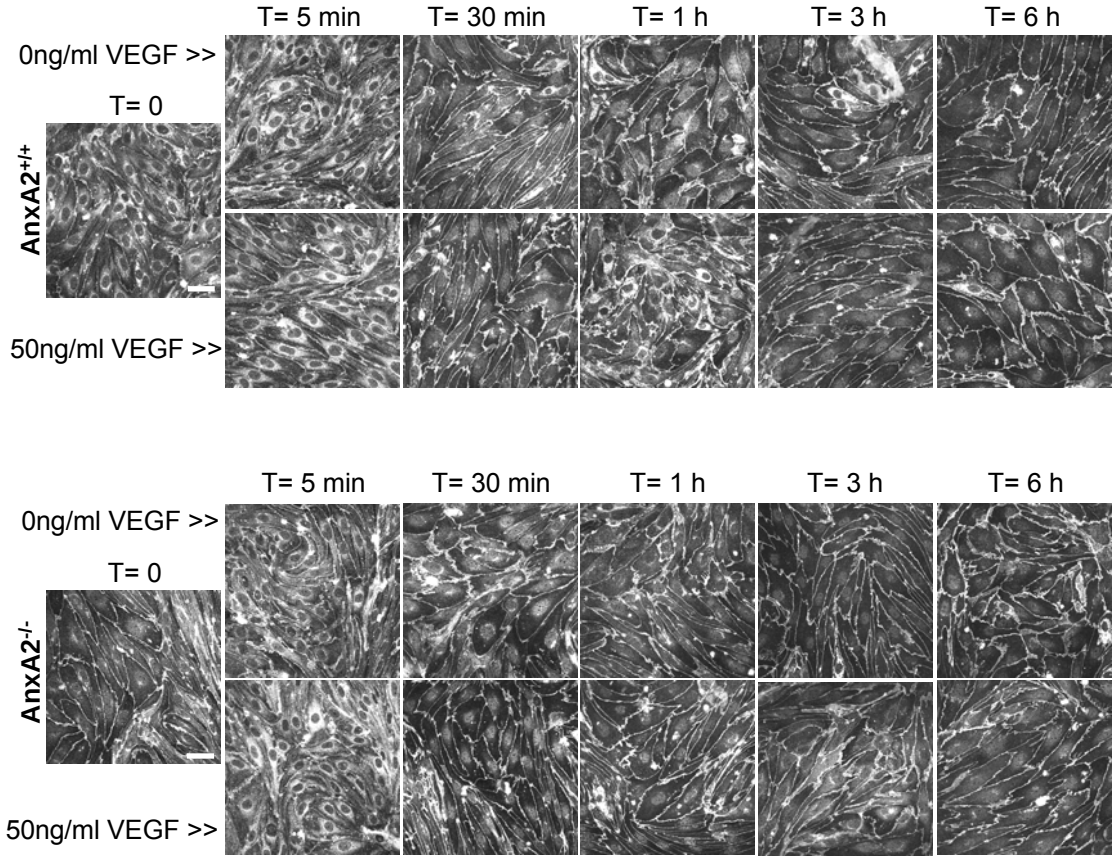

Figure 4.12: Enhanced sensitivity of VE-cadherin to VEGF in endothelial cells lacking annexin 2.

$AnxA2^{+/+}$ and $AnxA2^{-/-}$ primary endothelial cells were isolated and exposed to 50 ng/ml VEGF or vehicle (0.1% BSA, PBS) protocol as detailed in the materials and methods. Cells were then fixed in 4% PFA at the various time points indicated and stained for VE-cadherin using Anti-VE-cadherin (1:50) and Anti-goat-Alexafluor-488 (1:1000). Scale bar 50 μm

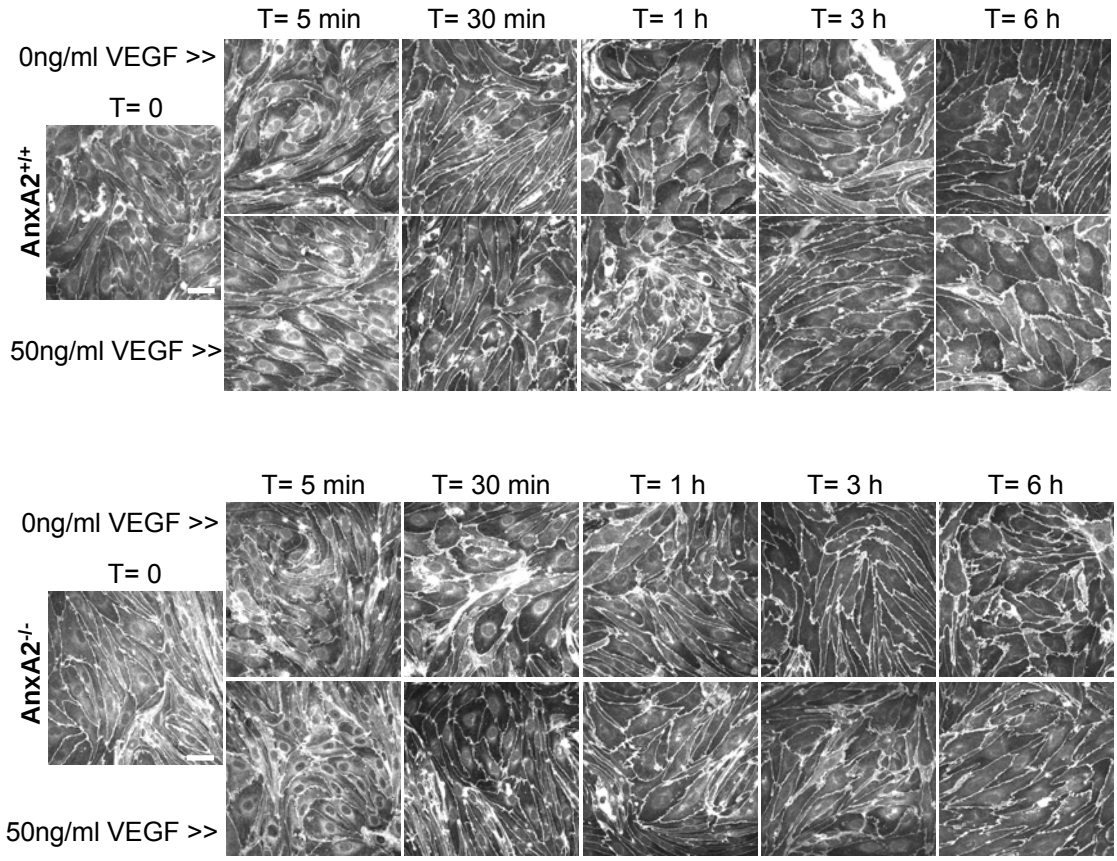

Figure 4.13: Effect of VEGF on β-catenin localisation in AnxA2$^{+/+}$ and AnxA2$^{-/-}$ endothelial cells.

AnxA2$^{+/+}$ and AnxA2$^{-/-}$ primary endothelial cells were isolated and exposed to 50 ng/ml VEGF or vehicle (0.1% BSA, PBS) protocol as detailed in the materials and methods. Cells were then fixed in 4% PFA at the various time points indicated and stained for β-catenin using Anti-β-catenin (1:100) and Anti-rabbit-Alexafluor-568 (1:1000). Scale bar 50 μm

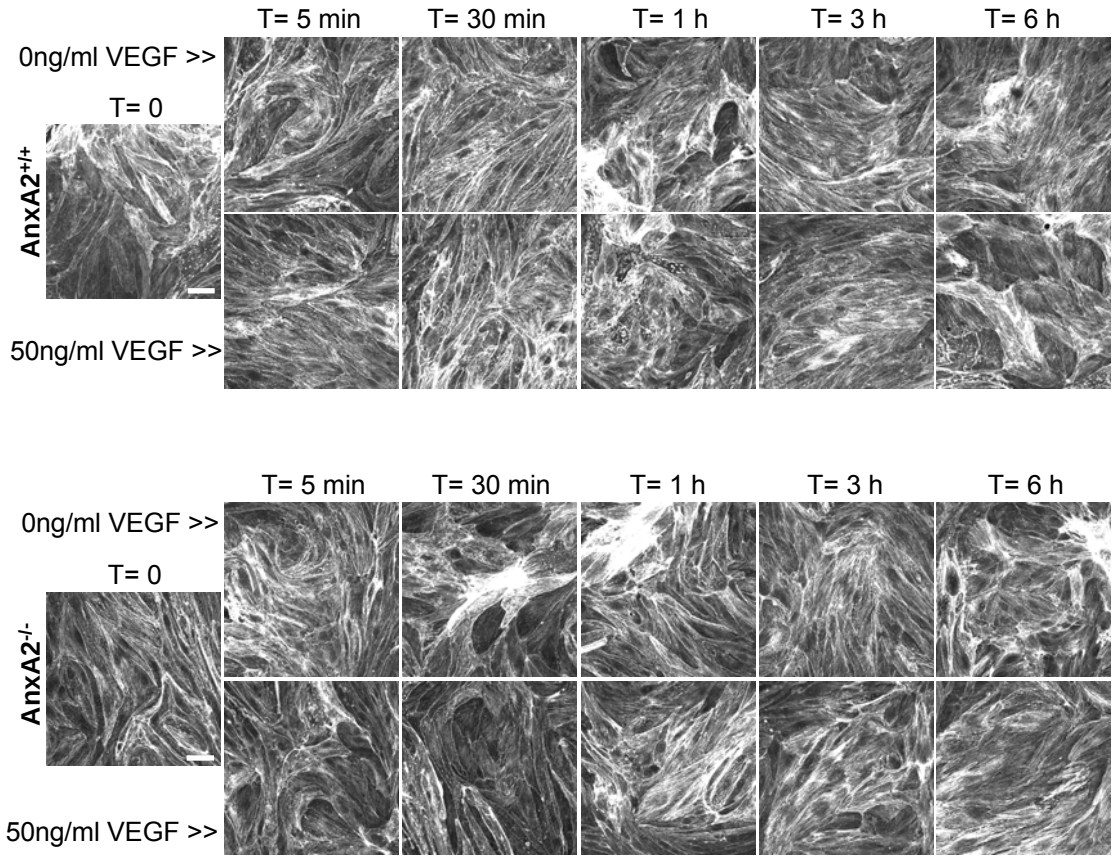

Figure 4.14: Effect of VEGF on F-actin localisation in AnxA2$^{+/+}$ and AnxA2$^{-/-}$ endothelial cells.

AnxA2$^{+/+}$ and AnxA2$^{-/-}$ primary endothelial cells were isolated and exposed to 50 ng/ml VEGF or vehicle (0.1% BSA, PBS) protocol as detailed in the materials and methods. Cells were then fixed in 4% PFA at the various time points indicated and stained for F-actin using Phalloidin-633 (1:60). Scale bar 50 μm

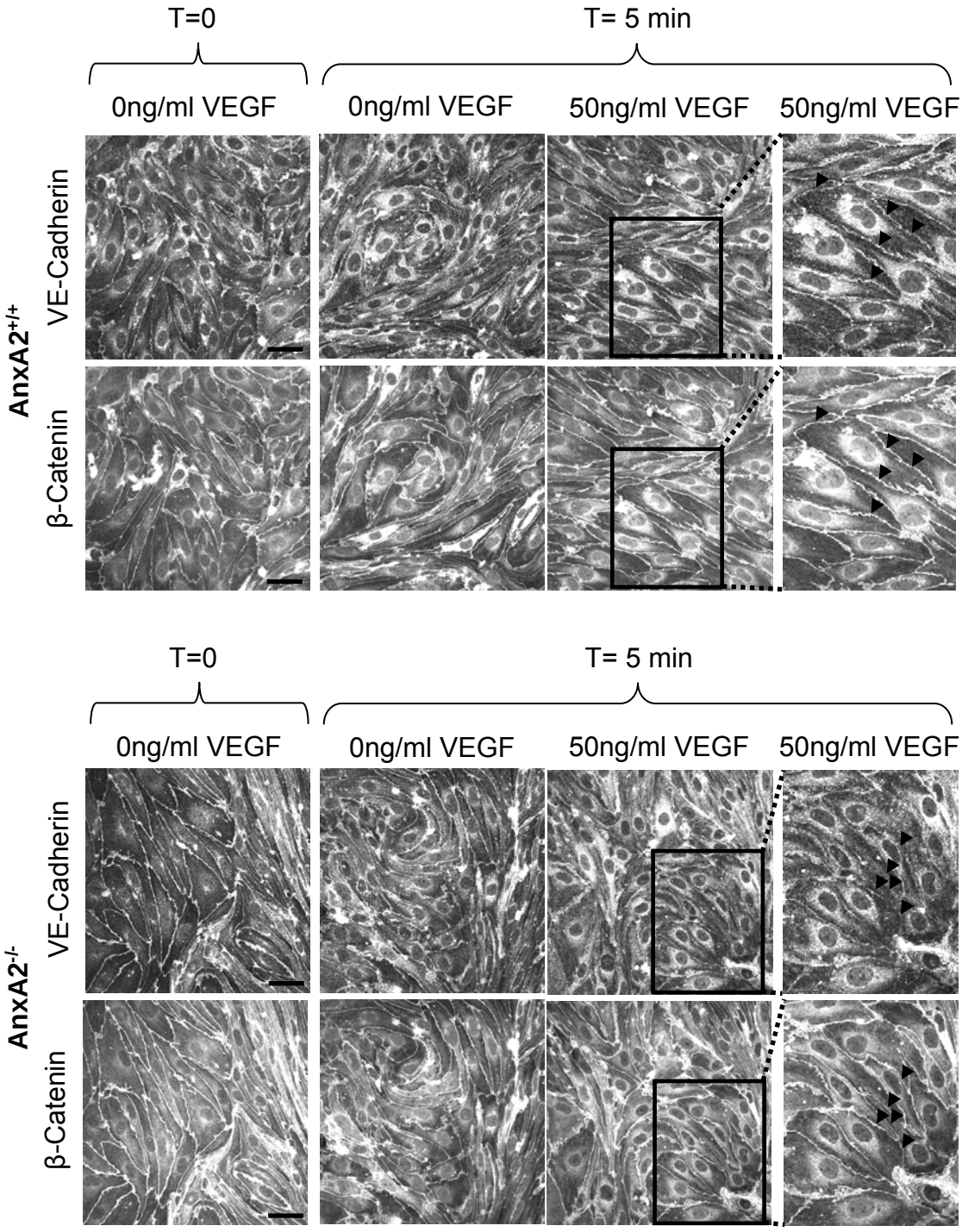

Figure 4.15: AnxA2$^{-/-}$ primary endothelial cells exposed to VEGF display changes in VE-cadherin localisation at 5 minutes.

AnxA2$^{+/+}$ and AnxA2$^{-/-}$ primary endothelial cells were isolated and exposed to 50 ng/ml VEGF or vehicle (0.1% BSA, PBS) protocol as detailed in the materials and methods. Cells were then fixed in 4% PFA at the various time points indicated and stained for VE-cadherin using Anti-VE-cadherin (1:50) and Anti-goat-Alexafluor-488 (1:1000). and B-catinin using Anti-β-catenin (1:100) and Anti-rabbit-Alexafluor-568 (1:1000).

with no significant differences detected. Having demonstrated that both AnxA2$^{+/+}$ and AnxA2$^{-/-}$ animals treated with streptozotocin have elevated blood glucose levels, it was decided to see what effect 16 weeks of *in vivo* hyperglycaemia would have on brain endothelial cell cultures. Consistent with the data in chapter 3, in non-diabetic animals the loss of annexin 2 was insufficient to disrupt the localisation of VE-cadherin, but cells contained more prominent actin stress fibres (Figure 4.16). Endothelial cells from diabetic mice differed in various respects. First, exposure to *in vivo* diabetic conditions resulted in substantially reduced yields from both AnxA2$^{+/+}$ and AnxA2$^{-/-}$ mice, since only small isolated patches of cells could be detected, rather than sheets of confluent endothelium. Nevertheless, there appeared to be no change in the distribution of F-actin attributable to diabetes, although any potential changes are more indicative of, or masked by the effect of, culture in sub-confluent conditions. Upon examination of AnxA2$^{-/-}$ endothelial cells extracted from diabetic mice the increase in actin stress fibre phenotype is still evident, however, in comparison to AnxA2$^{+/+}$ diabetic endothelial cells the distribution of VE-cadherin at some cell to cell contacts is discontinuous, suggesting a defect in junctional targeting (Figure 4.17, red arrows). These data would therefore suggest that the loss of annexin 2 in diabetic conditions may disrupt the localisation of VE-cadherin, however, further sampling of diabetic animals would be needed to provide statistical significance.

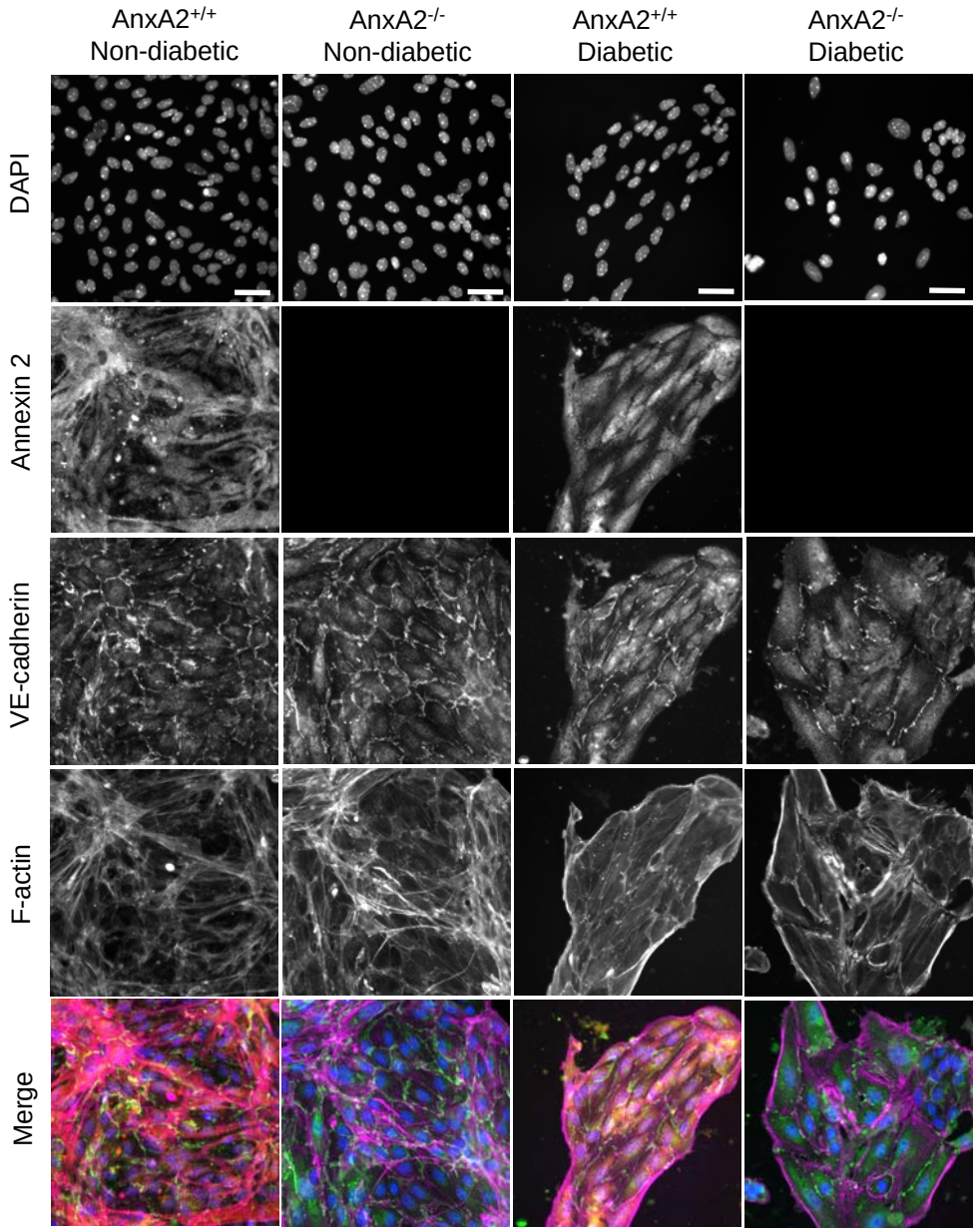

Figure 4.16: Assessment of VE-cadherin localisation in endothelial cells isolated from AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic and diabetic animals.
Endothelial cells were isolated from AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic and diabetic animals, 16 weeks post streptozotocin or sham treatment, as detailed in the materials and methods. Cells were then cultured for 1 week in either hyperglycaemic (25mM glucose) or euglycaemic (5mM glucose) conditions. Samples were then fixed in 4% paraformaldehyde and stained for DNA, VE-cadherin, F-actin and annexin 2 using DAPI (1:500), Anti-VE-cadherin (1:50), Annexin 2 (1:100; from Dr Jesus Ayala-Sanmartin) and Phalloidin-633 (1:60), Anti-Goat-Alexafluor-488 (1:1000), Anti-Rabbit-Alexafluor-568 (1:1000). Scale bar: 50 µm

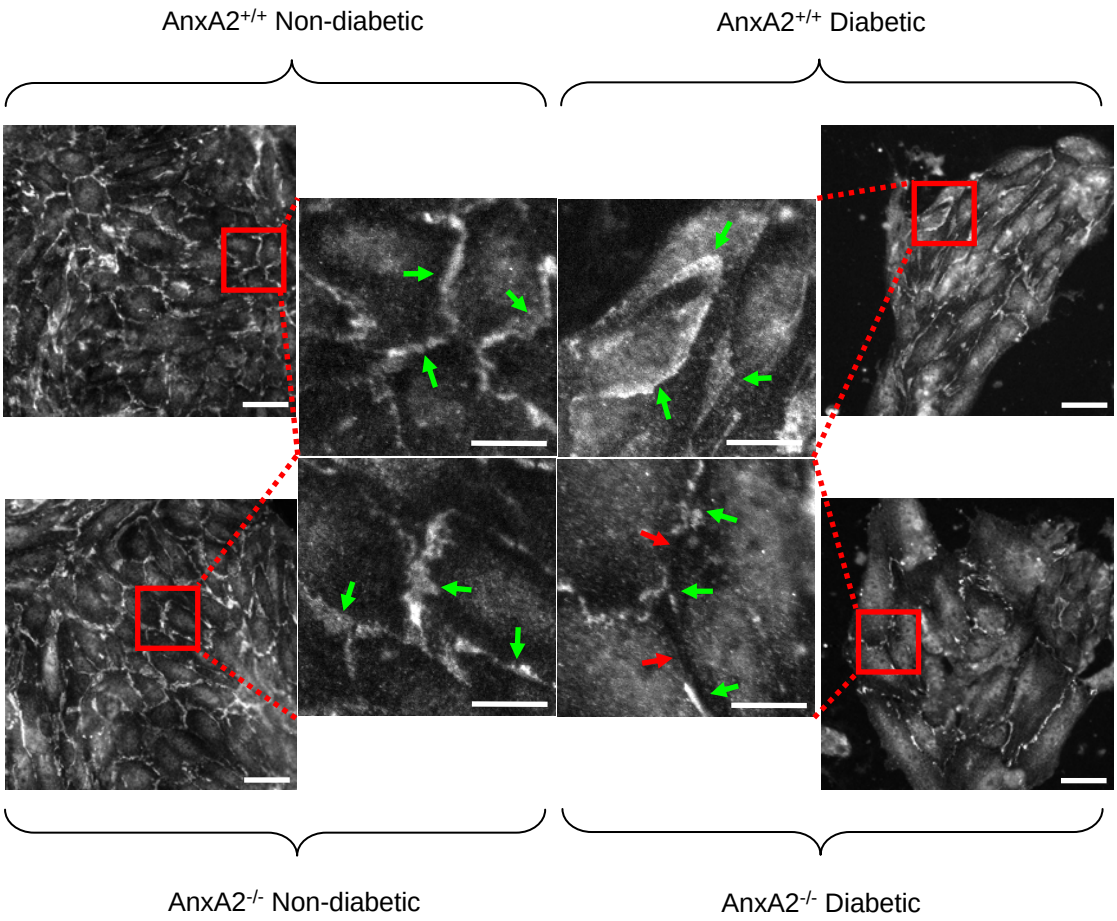

Figure 4.17: Assessment of VE-cadherin localisation in endothelial cells isolated from AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic and diabetic animals.
Endothelial cells were isolated from AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic and diabetic animals, 16 weeks post streptozotocin or sham treatment, as detailed in the materials and methods. Cells were then cultured for 1 week in either hyperglycaemic (25mM glucose) or euglycaemic (5mM glucose) conditions. Samples were then fixed in 4% paraformaldehyde and stained for VE-cadherin Anti-VE-cadherin (1:50) and Anti-Goat-Alexafluor-488 (1:1000). Areas of intact VE-cadherin staining are illustrated in green arrows whilst those in red depict areas where VE-cadherin is absent. Scale bar: outside panels = 50 µm , inside panels = 20µm.

Chapter 5 Results

Chapter 5 Results

Work in the previous chapter revealed that AnxA2$^{-/-}$ diabetic animals exhibit early indicators of diabetic retinopathy, and that cells lacking annexin 2 may have altered responses to VEGF. During the collection of data for blood glucose levels and weight, it was noticed that cages housing diabetic mice lacking annexin 2 needed cleaning more often than those of their wild type counterparts. Although increased urine output is a recognised consequence of uncontrolled diabetes, excessive urine output beyond that expected can be indicative of more severe problems. Diabetic kidney disease (Diabetic nephropathy) is characteristic of uncontrolled diabetes, culminates in decreased kidney function and has links with the incidence of diabetic retinopathy[403,404], hence we decided to examine whether the lack of annexin 2 accelerated the progression of diabetic nephropathy.

5.1 Assessment of polydipsia in diabetic mice

One of the symptoms of uncontrolled diabetes is excessive urination and hence excessive drinking or polydipsia. Since we had neither the permission nor equipment to measure urine production accurately, we instead measured the amount of water consumed by the various groups of mice over a 24 hour period. To achieve this, mice were housed either alone or in phenotypic groups and their water bottles weighed before and after a given 24 hour period. Both AnxA2$^{+/+}$ and AnxA2$^{-/-}$ mice without diabetes had a similar intake of water at approximately 6 ml in a 24 hour period (Figure 5.1). Diabetic mice had a 4-6 fold increase in their water intake over their control littermates (AnxA2$^{+/+}$ 26.7 ± 1.49 ml) (AnxA2$^{-/-}$ 33.7 ± 1.00 ml), with AnxA2$^{-/-}$ diabetic mice exhibiting a significant increase in the amount of water consumed, compared to their diabetic AnxA2$^{+/+}$ counterparts ($p<0.01$).

5.2 Assessment of microalbuminuria in diabetic mice

Having shown that AnxA2$^{-/-}$ diabetic mice display polydipsia, the presence of increased albumin in the urine, or microalbuminuria, was examined. Early in the day mice were handled over material that would collect any urine excreted as a normal consequence of their handling. Urine samples, 16 weeks post streptozotocin or sham

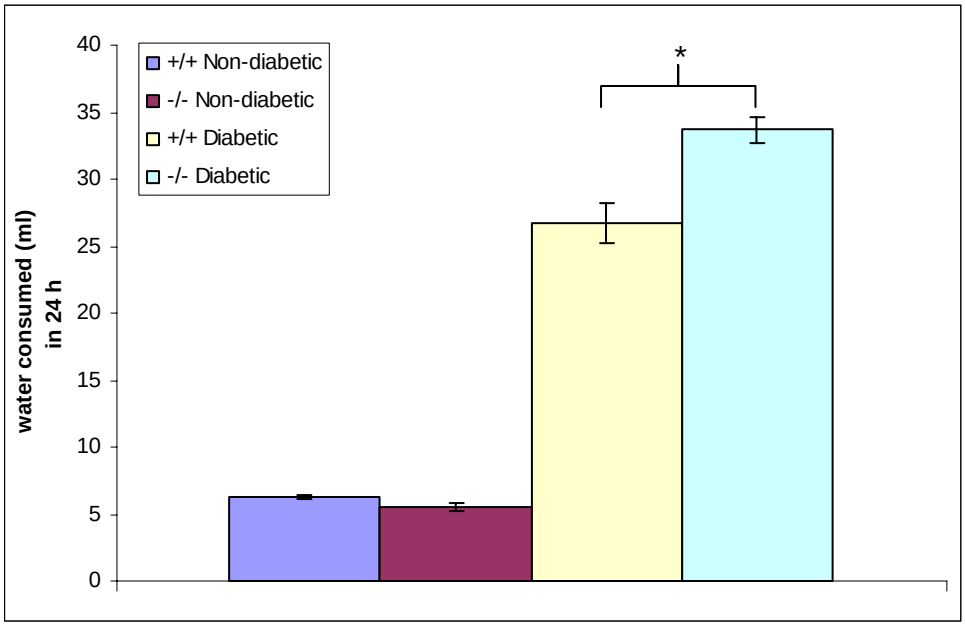

Fig 5.1: Assessment of water consumption in diabetic mice.
All mice examined for polydipsia had their water reservoirs weighed before and after a given 24 hour period to measure water intake as detailed in the materials and methods. Mean water volume consumed was then plotted on the graph shown. Error bars denote ±SEM, *=$p<0.01$.

treatment, were then tested for levels of albumin and creatinin by ELISA (Figure 5.2). The data show that the levels of albumin excreted by the AnxA2$^{+/+}$ control mice are within those expected for the c57bl/6 strain (77.6 ± 26.2 μg albumin / mg creatinin)[405]. Comparing this then to their diabetic counterparts a trend towards higher albumin excretion levels can be observed, although the differences are not statistically significant. Comparison of AnxA2$^{+/+}$ non-diabetic mice to AnxA2$^{-/-}$ non-diabetic mice reveals a similar trend, whereby albumin levels are increased in mice lacking annexin 2 to around twice the level of their wild-type counterparts (161.9 ± 74.8 μg albumin / mg creatinin), in contrast, the differences in this case are close to being significant ($p<0.077$). Interestingly, AnxA2$^{-/-}$ diabetic mice had the highest albumin excretion rate of all the mice (399.1 ± 195.1), which is significantly higher than the AnxA2$^{+/+}$ diabetic mice ($p<0.05$), indicating that mice lacking annexin 2 have increased albumin excretion than wild type mice, potentially exacerbated by some inherent kidney dysfunction induced by the loss of annexin 2.

5.3 Kidney weights of diabetic mice are increased

Having demonstrated potential kidney problems in the AnxA2$^{-/-}$ mice, at least at the level of albumin and urine excretion, and that the presence of diabetes exacerbates these changes, it was decided to examine the kidneys of these mice in more detail. Kidney weights of the mice were assessed, with comparisons to their final body weight to give a percentage of body weight (Figure 5.3). Both AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic mice yielded similar results, 1 ± 0.16% and 0.8 ± 0.11% respectively, indicating that the loss of annexin 2 does not influence kidney weight. When compared to diabetic mice, it was observed that AnxA2$^{-/-}$ diabetic mice had a significantly higher mean kidney weight than their non-diabetic littermates (1.39 ± 0.28%) ($p<0.05$) as did AnxA2$^{+/+}$ diabetic mice (1.36 ± 0.17%) ($p=0.05$). Interestingly there was no significant difference between AnxA2$^{+/+}$ and AnxA2$^{-/-}$ diabetic mice, indicating that although the presence of diabetes was sufficient to induce changes in kidney weight the loss of annexin 2 did not exacerbate or attenuate this effect.

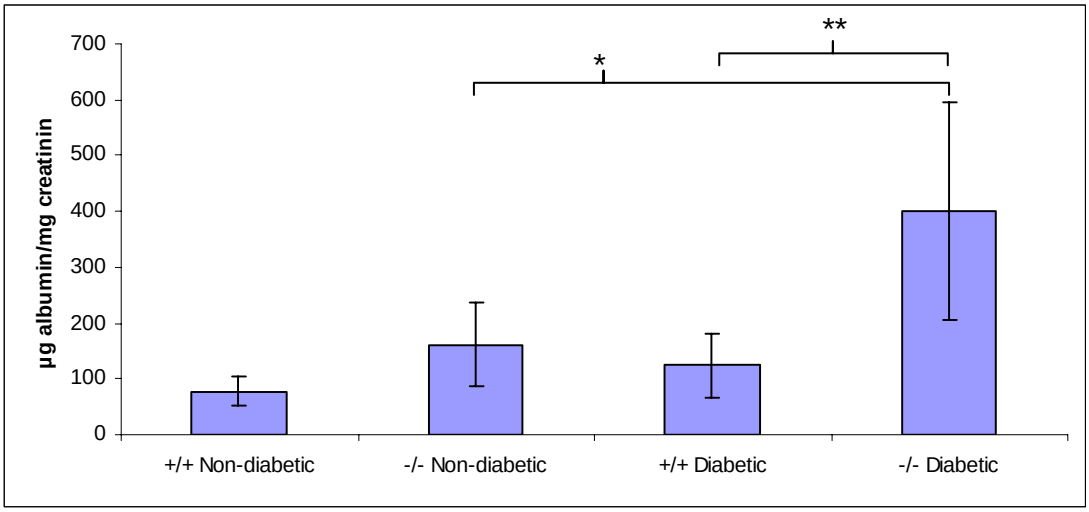

Fig 5.2: Assessment of microalbuminuria in diabetic mice.
Urine samples were collected from AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic and diabetic mice 16 weeks post streptozotocin treatment and subjected to albumin and creatinin ELISAs as detailed in the materials and methods. Data were then plotted from n=4 (AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic) or n=5 (AnxA2$^{+/+}$ and AnxA2$^{-/-}$ diabetic) mice as mean µg albumin / mg creatinin ± SD. *=$p<0.06$ **=$p<0.05$.

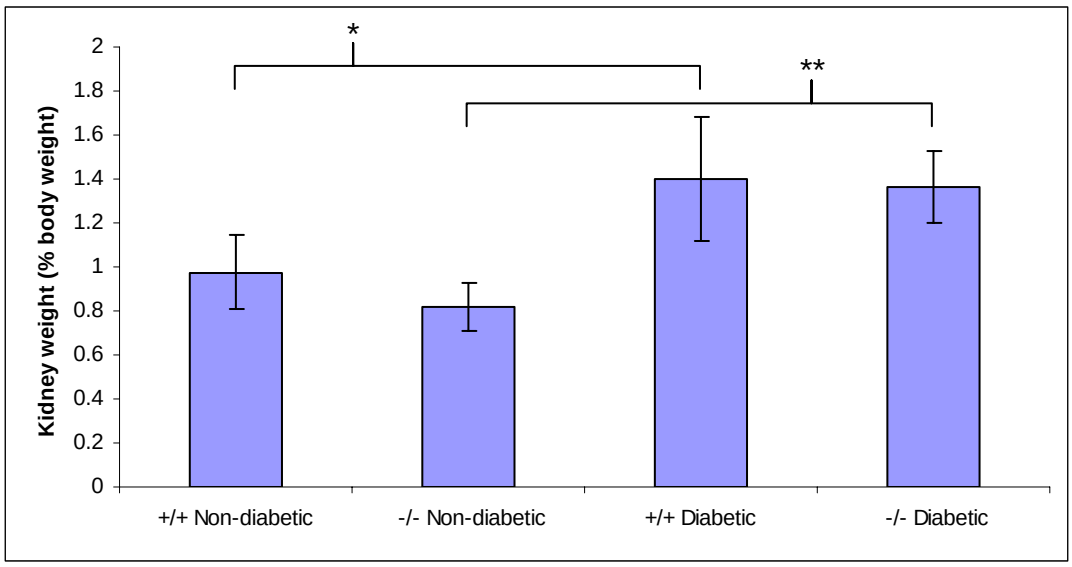

Fig 5.3: Assessment of kidney weights in diabetic mice.
Kidneys from mice were weighed and compared to the final weight recording of the mouse to give a percentage weight value. Error bars denote ± SD, $3 \leq n \leq 8$. *=p=0.05, **=$p<0.05$

5.4 Haematoxylin and eosin (H&E) staining of kidney reveals AnxA2$^{-/-}$ diabetic animals have aggravated diabetic nephropathy

To obtain an overall view of the various anatomical regions of the kidney 6μm sections were taken from paraffin embedded tissue and stained with H&E (Figure 5.4). Each set of images consists of a gross low magnification view from a midline coronal section, with higher magnification images from different areas of the kidney: cortex, medulla, calyces / renal pelvis and glomeruli. Both AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic mice display a normal anatomical structure of the kidney, with the various regions clearly defined and no malformations detectable. This indicates that the absence of annexin 2 is not sufficient to cause any obvious problems in the general structure of the kidney. However, if images of kidney sections obtained from AnxA2$^{+/+}$ non-diabetic mice are compared with those of the AnxA2$^{+/+}$ diabetic mice some minor differences become apparent. Tubules in the cortex region appear mildly dilated, the tubules of the medulla also seem less uniform in their distribution, potentially indicating that some have been lost, and tubules of the calyces also appear less organised and uniform. These differences however are minor compared to those observed in the AnxA2$^{-/-}$ diabetic kidney, where there is substantial dilation of the tubules in all the various regions, with the different regions harder to identify since the general organisation of the kidney is severely compromised. With these indications of increased diabetic nephropathy noted a further staining regimen was employed to visualise more clearly the apparent changes.

5.5 Periodic acid Schiff staining in AnxA2$^{-/-}$ diabetic animals

In addition to general morphological changes observed by H&E staining, changes evident in diabetic kidney tissue can be observed more clearly using periodic acid Schiff (PAS) stain. Hence similar kidney sections to those that were stained with H&E were also stained with PAS to highlight these changes (Figure 5.5). PAS staining confirms that AnxA2$^{-/-}$ diabetic mice have an accentuated response to diabetes, whilst AnxA2$^{-/-}$ control mice appear to have normal kidney architecture. The advantage with the PAS stain is that the basement membranes of the tubules and glomeruli are more readily visible as red lines on the images. Some distinction was also possible between the epithelial cells and their microvillar extensions that project into the lumen. Qualitative evaluation of these images reveals that AnxA2$^{-/-}$ diabetic animals have reduced tubular cell thickness in comparison to their control littermates and

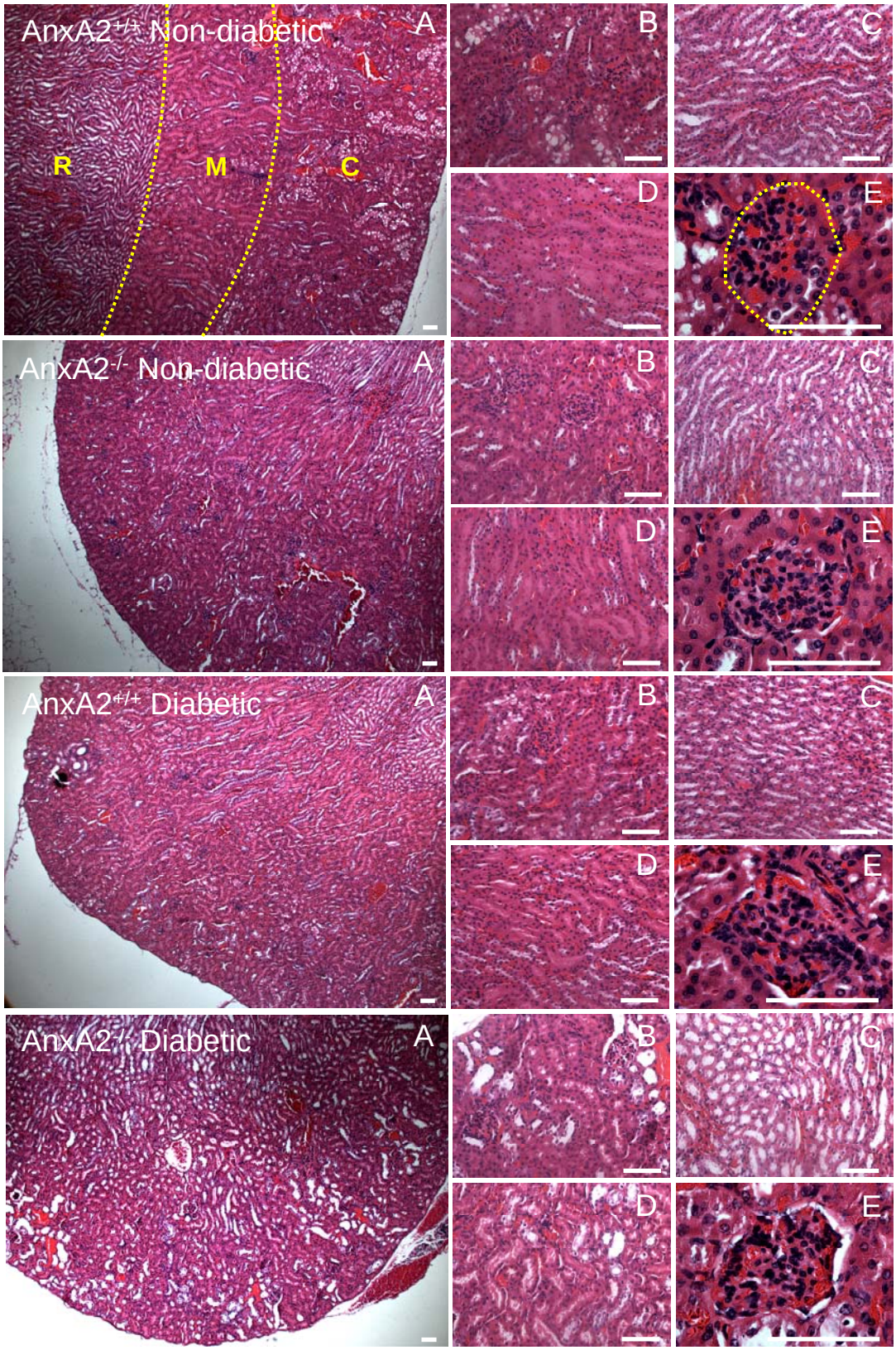

Fig 5.4: H&E staining of kidneys reveals indications of accentuated diabetic nephropathy in AnxA2$^{-/-}$ diabetic mice. Kidneys were isolated, fixed, embedded and sectioned as detailed in the materials and methods. H&E staining was applied also as detailed in the material and methods with, representative images of 3 separate samples shown above. Panel A shows an overall image of the kidney, B shows the cortex region, D the medulla and C the calyx / renal pelvis, with panel E showing a single glomerulus. Different regions are illustrated in AnxA2$^{+/+}$ Non-diabetic C=Cortex, M=Medulla, R=Calyx/Renal pelvis, yellow outline in panel E indicates glomerulus . Scale bar in all images = 100 µm.

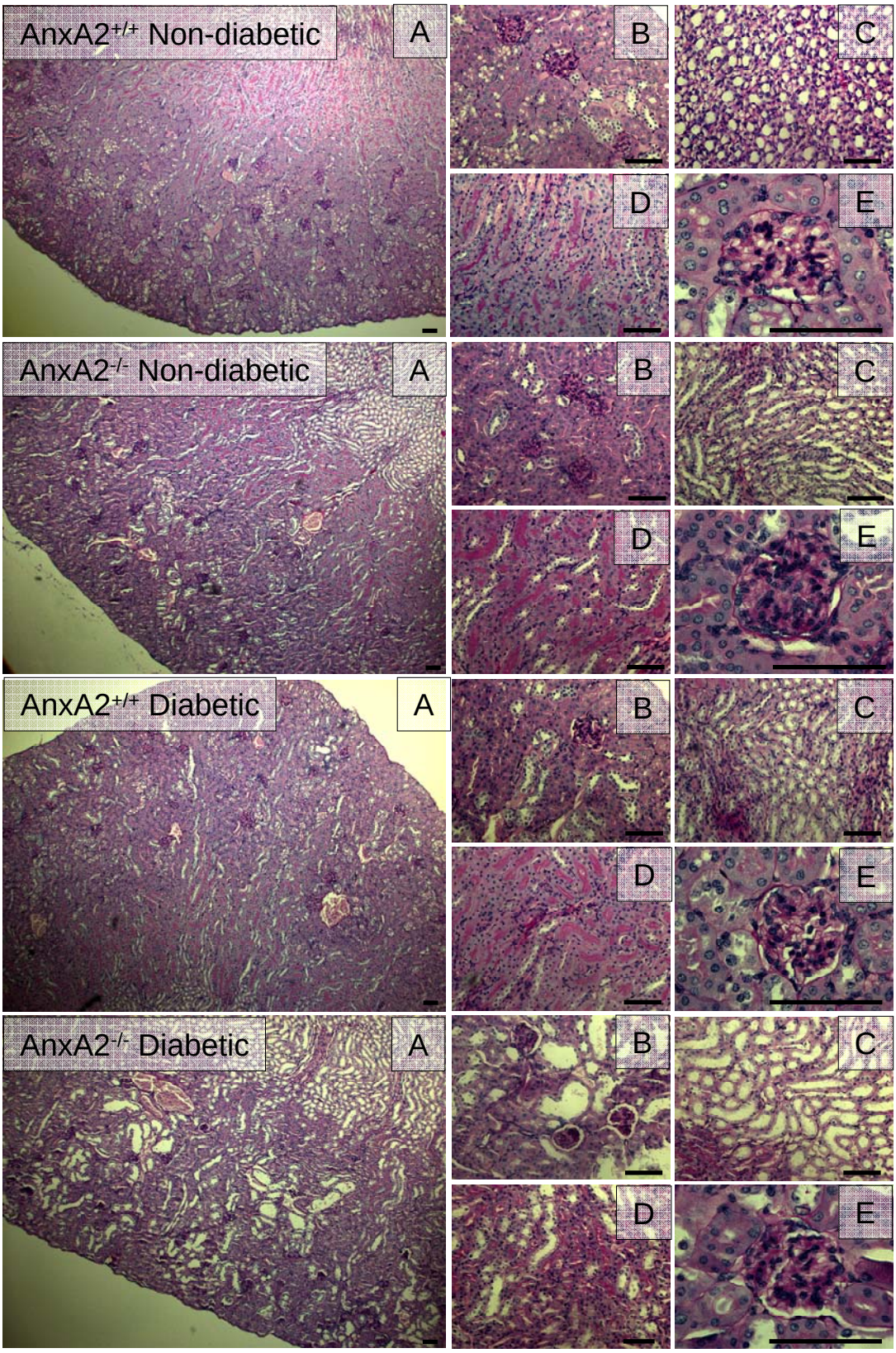

Fig 5.5: PAS staining of kidneys reveals indications of accentuated diabetic nephropathy in AnxA2$^{-/-}$ diabetic mice. Kidneys were isolated, fixed, embedded and sectioned as detailed in the materials and methods. PAS staining was applied also as detailed in the material and methods with representative images of 3 separate samples shown above. Panel A shows an overall image of the kidney, B shows the cortex region, D the medulla and C the calyx / renal pelvis, with panel E showing a single glomerulus. Scale bar in all images represents 100 µm

AnxA2$^{+/+}$ mice. To attempt to assess the degree of cellular thinning 300 cells from three individual animals were quantified and lengths measured. In comparison to the AnxA2$^{+/+}$ non-diabetic mice the data show that AnxA2$^{-/-}$ non-diabetic mice have a slightly reduced tubular cell thickness, and that diabetic AnxA2$^{+/+}$ and AnxA2$^{-/-}$ tubular cell thickness is significantly less than the non-diabetic controls ($p<0.05$) (Figure 5.6). In addition, AnxA2$^{-/-}$ diabetic mice display a slightly reduced cell thickness in comparison to the AnxA2$^{+/+}$ diabetic counterparts, though the difference is not significant. A further advantage of staining kidney sections with PAS is that any potential mesangial matrix expansion can be detected. Assessment of mesangial matrix content was made by measurement of the percentage area of intensely PAS positive extravascular region in each glomerulus, not including Bowman's space. Both AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic animals display a similar level of mesangial matrix with approximately 5% of the total extravascular area intensely PAS positive (Figure 5.7). In addition, both AnxA2$^{+/+}$ and AnxA2$^{-/-}$ diabetic animals show significantly more PAS positive regions than their non-diabetic littermates ($p<0.05$), demonstrating that diabetes induces an increase in mesangial matrix. Furthermore AnxA2$^{-/-}$ diabetic mice have significantly more mesangial matrix than AnxA2$^{+/+}$ diabetic mice ($p<0.01$), demonstrating that the absence of annexin 2 exacerbates the accumulation of this matrix in diabetes.

5.6 Electron microscopy reveals basement membrane thickening in diabetic mice

Although PAS staining illustrates the basement membrane and mesangial matrix in the kidney better than H&E staining, to get an accurate measurement of the thickness of the basement membrane higher magnification are resolution are required, and hence electron microscopy was employed. Kidney cortex samples from AnxA2$^{+/+}$ and AnxA2$^{-/-}$ control and diabetic mice were processed for electron microscopy, and examined on a JEOL 1010 transmission electron microscope.

Inspection of the general architecture of the tubular cell reveals that the loss of annexin 2 is insufficient to cause any gross ultrastructural changes, in particular the apical villi of the tubular epithelial cells are apparently normal (Figure 5.8). Normal cellular ultrastructure was also observed in the majority of both the AnxA2$^{+/+}$ and AnxA2$^{-/-}$ diabetic samples (Figure 5.9). However, in these samples there were instances where the consequences of diabetes were evident in reduced cell thickness, and reduced villi length (Figure 5.10). In addition to examination of general architecture, basement

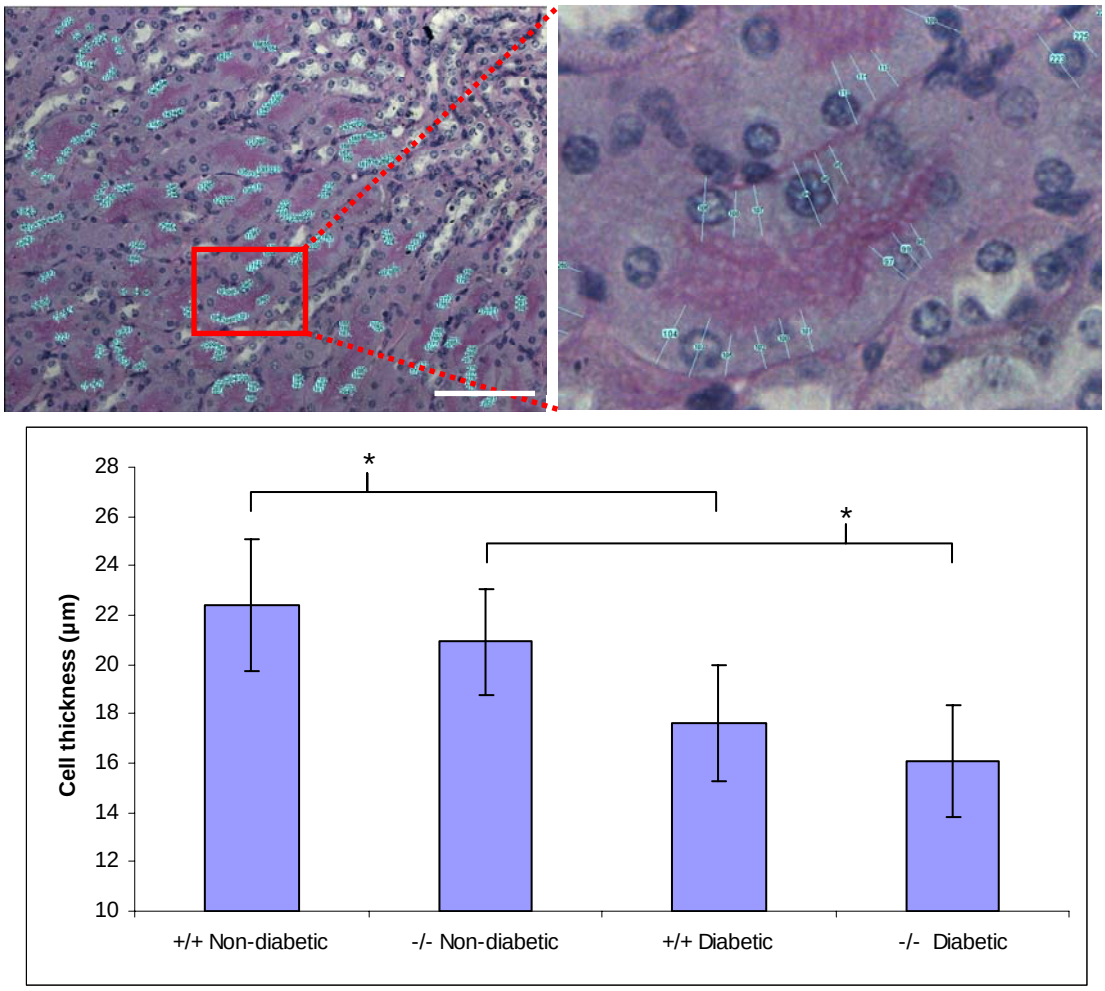

Fig 5.6: AnxA2$^{-/-}$ diabetic mice show reduced epithelial cell thickness.
Images of kidneys stained with PAS were taken from both AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic and diabetic mice and subjected to cell thickness analysis as demonstrated above using Image-J software. Mean thickness was then plotted on the graph shown with error bars denoting ±SEM. Scale bar represents 100µm. *=$p<0.05$. n=300 (100 cells quantified from 3 individual mice)

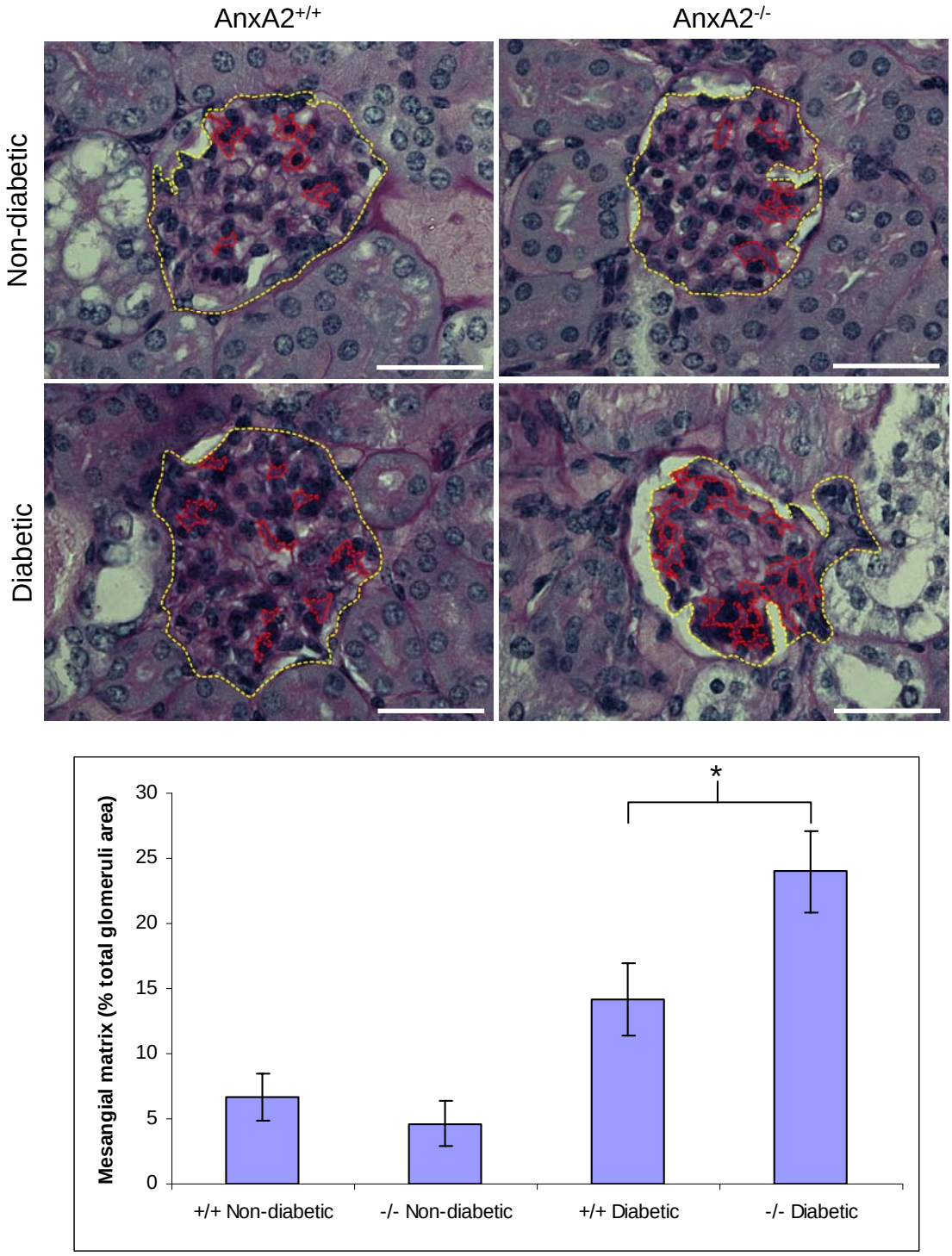

Fig 5.7: Analysis of mesangial matrix expansion in diabetic mice.
Images of kidneys stained with PAS were taken from both AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic and diabetic mice and analysed for amount of mesangial matrix, seen as an intense PAS stain. Representative images are shown of AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic and diabetic above with yellow dotted lines denoting the glomerulus and red dotted lines the mesangial matrix. Red areas were then collated and expressed as a percentage of total glomerula area. The graph shows the mean area of mesangial matrix of each animal group ± SD. *= $p<0.01$.

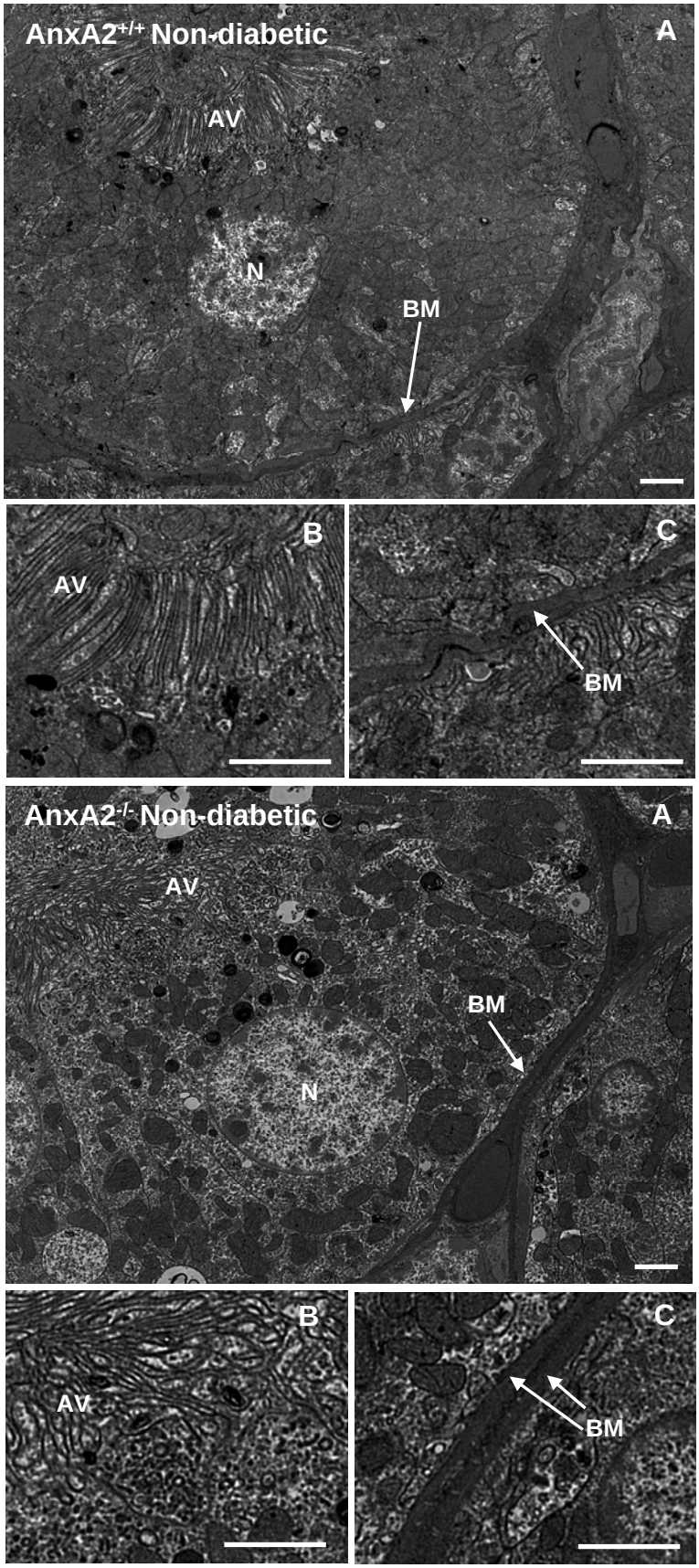

Fig 5.8 Examination of kidney tubular cells by electron microscopy.
Kidney samples were prepared for imaging by electron microscopy, as detailed in the materials and methods. Samples were prepared from AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic. In each group of images A is an overview of the tubule cell whilst B and C give views of the apical villi and basement membrane respectively. N=nucleus, BM= Basement membrane, AV=apical villi. Scale bar = 2μm.

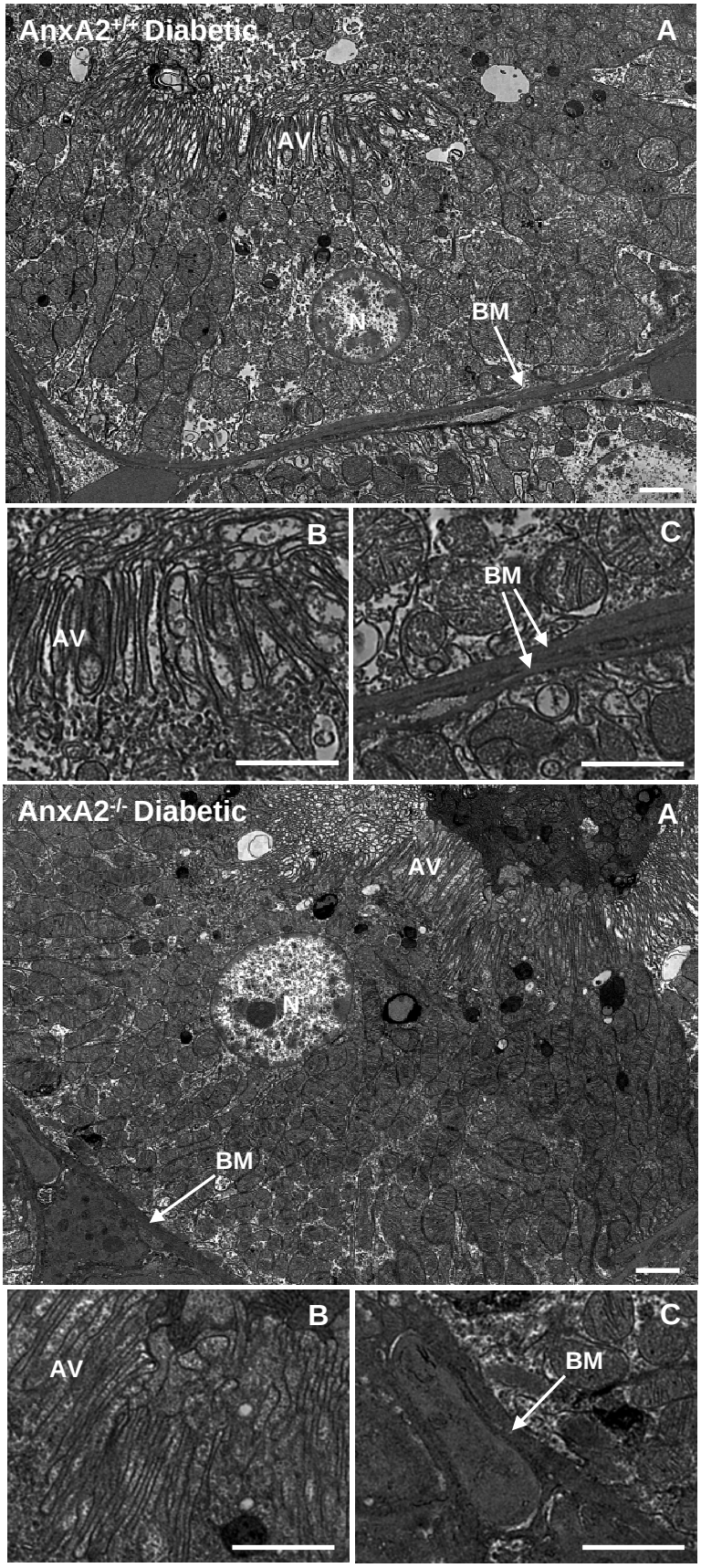

Fig 5.9 Examination of kidney tubular cells by electron microscopy.
Kidney samples were prepared for imaging by electron microscopy, as detailed in the materials and methods. Samples were prepared from AnxA2$^{+/+}$ and AnxA2$^{-/-}$ Diabetic. In each group of images A is an overview of the tubule cell whilst B and C give views of the apical villi and basement membrane respectively. N=nucleus, BM= Basement membrane, AV=apical villi. Scale bar = 2µm.

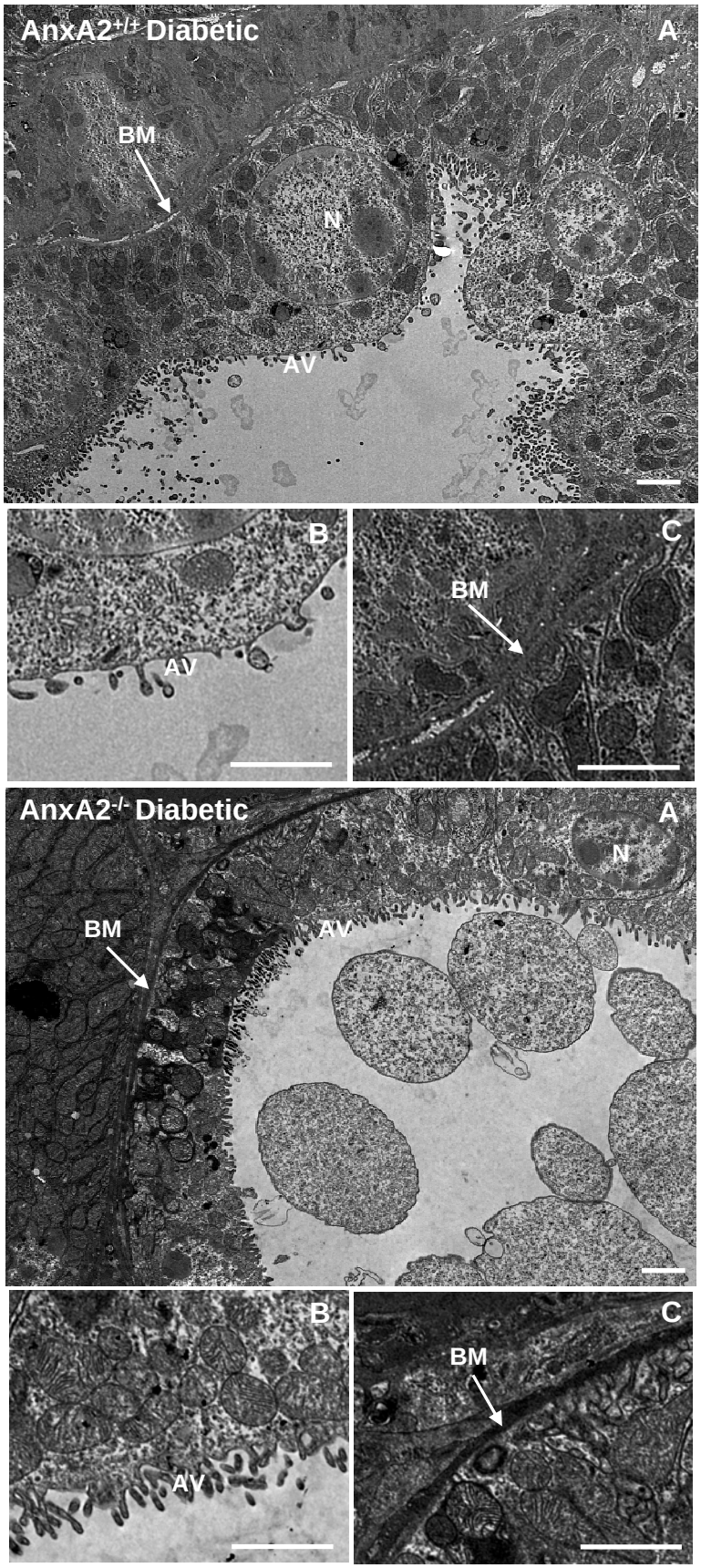

Fig 5.10 Examination of kidney tubular cells by electron microscopy.
Kidney samples were prepared for imaging by electron microscopy, as detailed in the materials and methods. Samples were prepared from AnxA2$^{+/+}$ and AnxA2$^{-/-}$ Diabetic. In each group of images A is an overview of the tubule cell whilst B and C give views of the apical villi and basement membrane respectively. N=nucleus, BM= Basement membrane, AV=apical villi. Scale bar = 2µm.

membrane thicknesses were measured from all the groups of mice. This analysis reveals the presence of some degree of basement membrane thickening in both the AnxA2$^{+/+}$ and AnxA2$^{-/-}$ diabetic animals compared to their non-diabetic littermates (Figure 5.11). However, the extent of this change was not statistically significant, and the degree of thickening in AnxA2$^{+/+}$ and AnxA2$^{-/-}$ diabetic mice was broadly similar. The tubular basement membrane is not however the principle site of thickening and hence capillary basement membranes of the glomeruli were also examined. Figure 5.12 shows a representative example from each of the groups of mice that were used for the quantification, with the lumen of the capillary evident in the centre of the image surrounded by fenestrated endothelium, basement membrane, and podocyte foot processes. In addition to measuring basement membrane thickness, it was also possible to detect potential changes in the endothelial and podocyte cell layers. Examining first the endothelium, in all the images from the four groups the endothelium appears fenestrated, suggesting that the absence of annexin 2 or the presence of diabetes does not alter this defining characteristic. The endothelium also appears continuous around the entire capillary, again suggesting that no significant loss of endothelial cells occurs as a result of diabetes or loss of annexin 2.

Examination of podocytes revealed a slightly different result. Firstly, podocytes in AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic mice appeared similar with even spacing between podocyte feet, and normal effacement of those feet to the basement membrane. Changes between the AnxA2$^{+/+}$ non-diabetic and AnxA2$^{+/+}$ diabetic images are apparent however, with fewer podocyte feet and increased levels of effacement (Figure 5.12, red arrows), suggesting changes consistent with diabetic nephropathy. Examining next the AnxA2$^{-/-}$ non-diabetic and diabetic images, similar changes in the podocyte feet are apparent, suggesting that these cells have undergone comparable disease-induced modifications, although whether the extent of change between the AnxA2$^{+/+}$ and AnxA2$^{-/-}$ diabetic animals is different is not easily assessed via electron microscopy alone, and would require further studies. Returning to the issue of basement membrane changes, the average thickness was quantified for a number of different capillary images for all four groups of mice. Both AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic mice exhibited similar thickness of the basement membrane (Figure 5.10). However, more substantial differences were observed in mice exposed to diabetes, both AnxA2$^{+/+}$ and AnxA2$^{-/-}$, with similar levels of thickening apparent, demonstrating that although diabetes is sufficient to drive an increase in basement membrane thickness, loss of annexin 2 does not compound that effect.

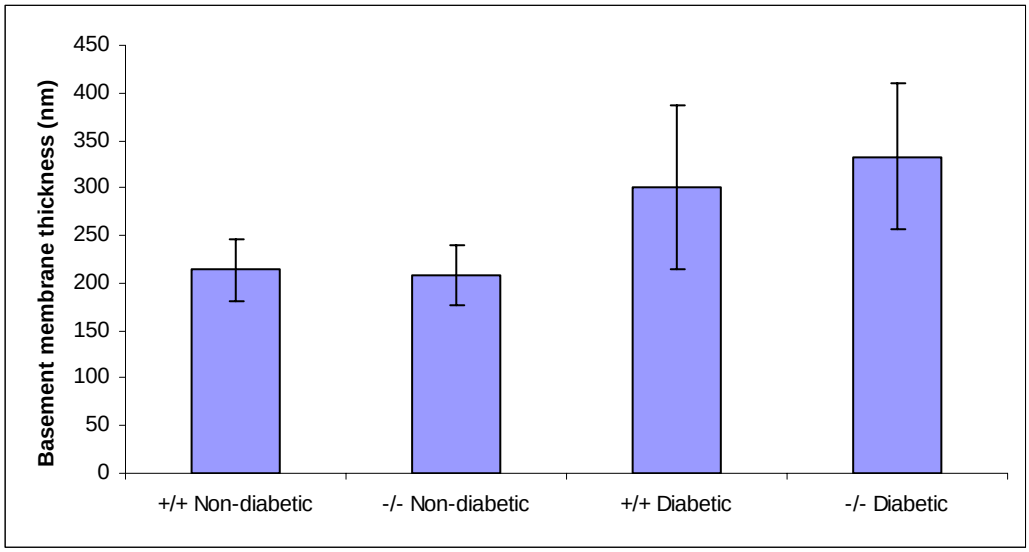

Fig 5.11: Diabetic mice show increased tubular basement membrane thickness.
Electron microscopy images were taken of kidney samples, and tubular basement membrane thickness quantified using Image-J. The graph shows mean value of all the measurements taken for each group ± SD, n=60 (30 measurements from 2 mice).

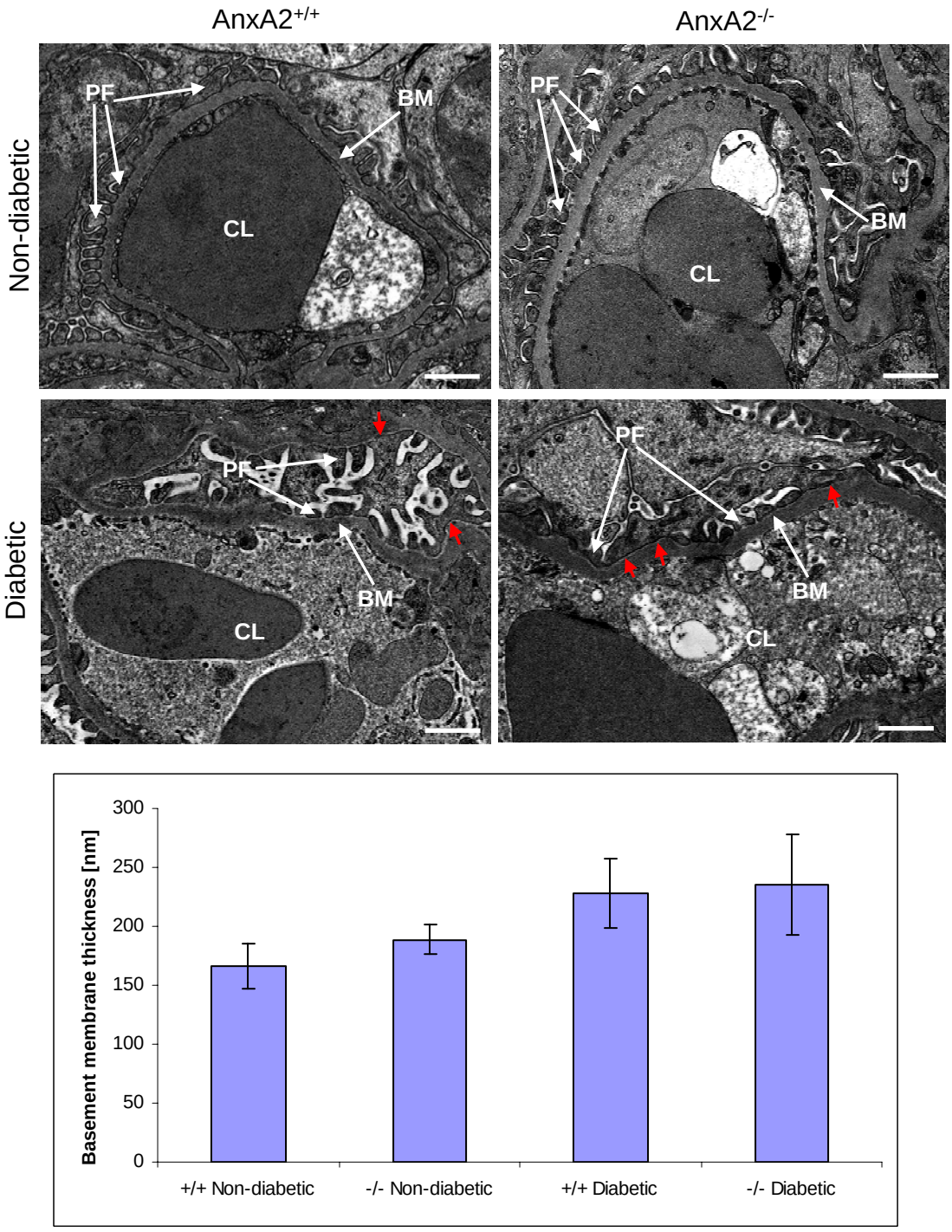

Fig 5.12: AnxA2$^{-/-}$ and AnxA2$^{+/+}$ diabetic mice show increased glomerular capillary basement membrane thickness.

EM images were taken from the various groups of mice and basement membrane thickness quantified using Image-J. The graph shows mean value of all the measurements taken for each group ± SD, n=60 (30 measurements taken from 2 mice). CL=Capillary lumen, BM=Basement membrane, PF=Podocyte feet, Red arrows=Podocyte foot effacement. Scale bar = 1 µm

5.7 Diabetic mice show intact endothelial junctions *in vivo*

Since most of the data in this chapter indicate problems with the glomeruli of mice exposed to diabetes that may be exacerbated by the loss of annexin 2, confocal microscopy was used to ascertain whether vascular endothelial cell junctional markers were affected in these mice. Kidneys from each group of mice were cryosectioned and stained for VE-cadherin, ZO-1 and F-actin, and projection images of glomeruli generated. Examining initially the staining for AnxA2$^{+/+}$ control mice (Figure 5.13), VE-cadherin staining was observed throughout the glomerulus, with particular areas of enrichment evident, presumably at endothelial cell junctions. Some staining of the surrounding tissue was also observed, including the nuclei, which was not expected, and since VE-cadherin has not been localised to the nucleus, this staining was considered likely to be artefactual. ZO-1 staining was also observed throughout the glomerulus, again with specific sites of enrichment, also presumably at the interfaces between cells. Interestingly, the sites of ZO-1 enrichment do not overlap with those of VE-cadherin, suggesting that the cells staining strongest for ZO-1 may be interstitial cells such as podocytes and mesangial cells. F-actin staining was observed throughout the glomerulus with no specific enrichments. Examining the differences between the other groups of mice, in all cases glomeruli were positive for VE-cadherin, ZO-1 and F-actin, with distributions similar to that of AnxA2$^{+/+}$ non-diabetic mice, indicating that either the actions of diabetes or loss of annexin 2 were not sufficient to cause any changes in the localisation of these proteins. While images were being collected for the examination of glomeruli, it was noted that areas of vasculature were easily identifiable. Figure 5.14 shows that VE-cadherin staining is clearly evident in all images across the full range of mice, with no change in its distribution as a consequence of loss of annexin 2 or the actions of diabetes. ZO-1 staining was also evident in all images, but a much lesser extent that VE-cadherin. F-actin staining was evident in all samples and illustrates the supporting cells that are wrapped around the vessel.

5.8 Annexin 2 is upregulated in response to diabetes

Because the antibodies available to stain annexin 2 were raised in mice, imaging of annexin 2 in murine kidney and eye produced unconvincing results. Other antibodies were therefore also tested on kidney sections, but similarly failed to yield images of sufficient quality for analysis (data not shown). To assess the effect of diabetes on

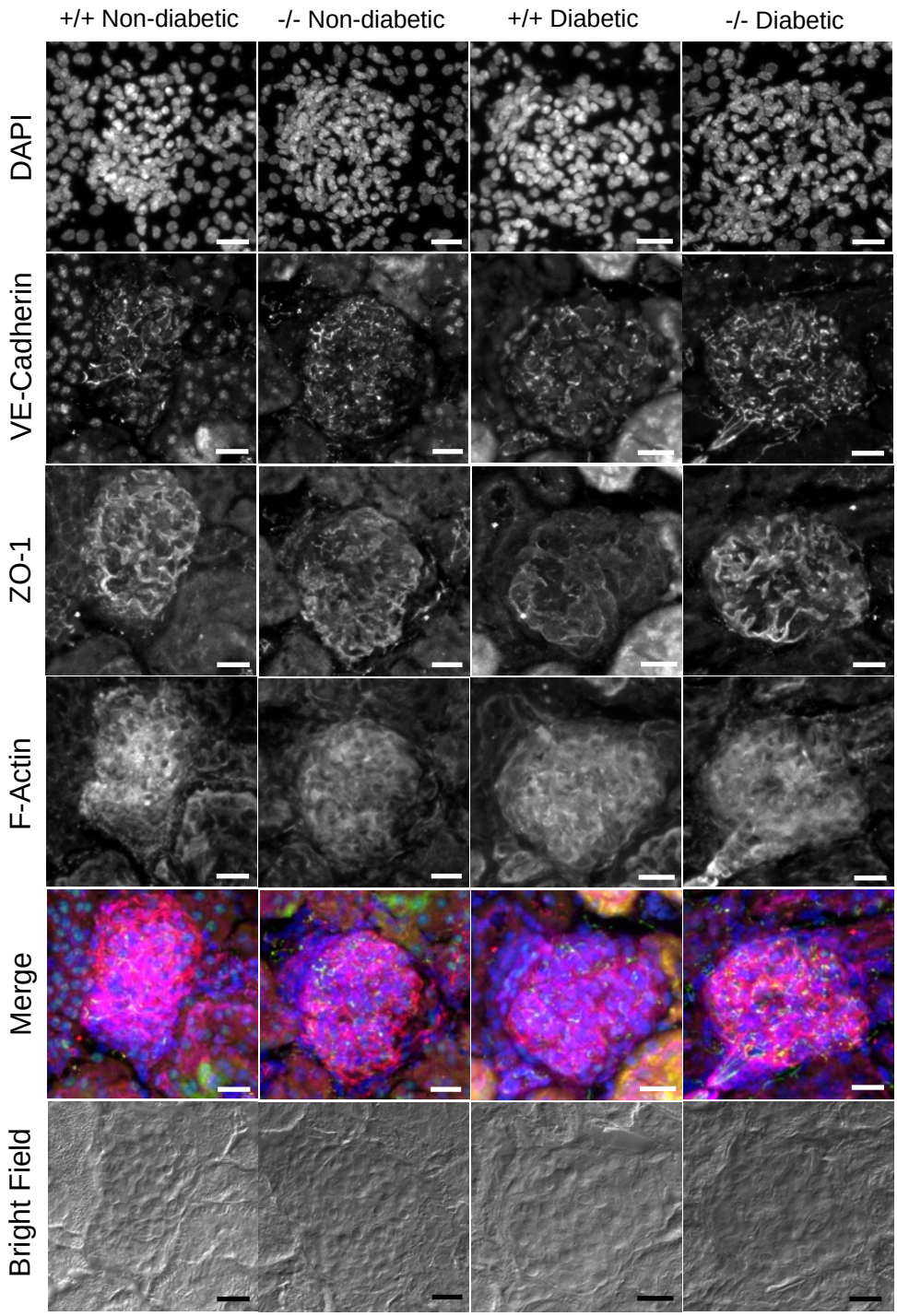

Fig 5.13: Assessment of glomerular junctional protein localisation.
Kidney tissue was sectioned, fixed and stained for analysis by confocal microscopy as detailed in the materials and methods. Glomeruli were then imaged, with a representative from each group of mice shown here, sections were stained using antibodies and reagents: DAPI (1:500), Anti-VE-cadherin (1:50), Anti-ZO-1 (1:300), Phalloidin-633 (1:60), Anti-Goat-Alexafluor-488 (1:1000), Anti-Rabbit-Alexafluor-568 (1:1000). Scale bar = 20µm

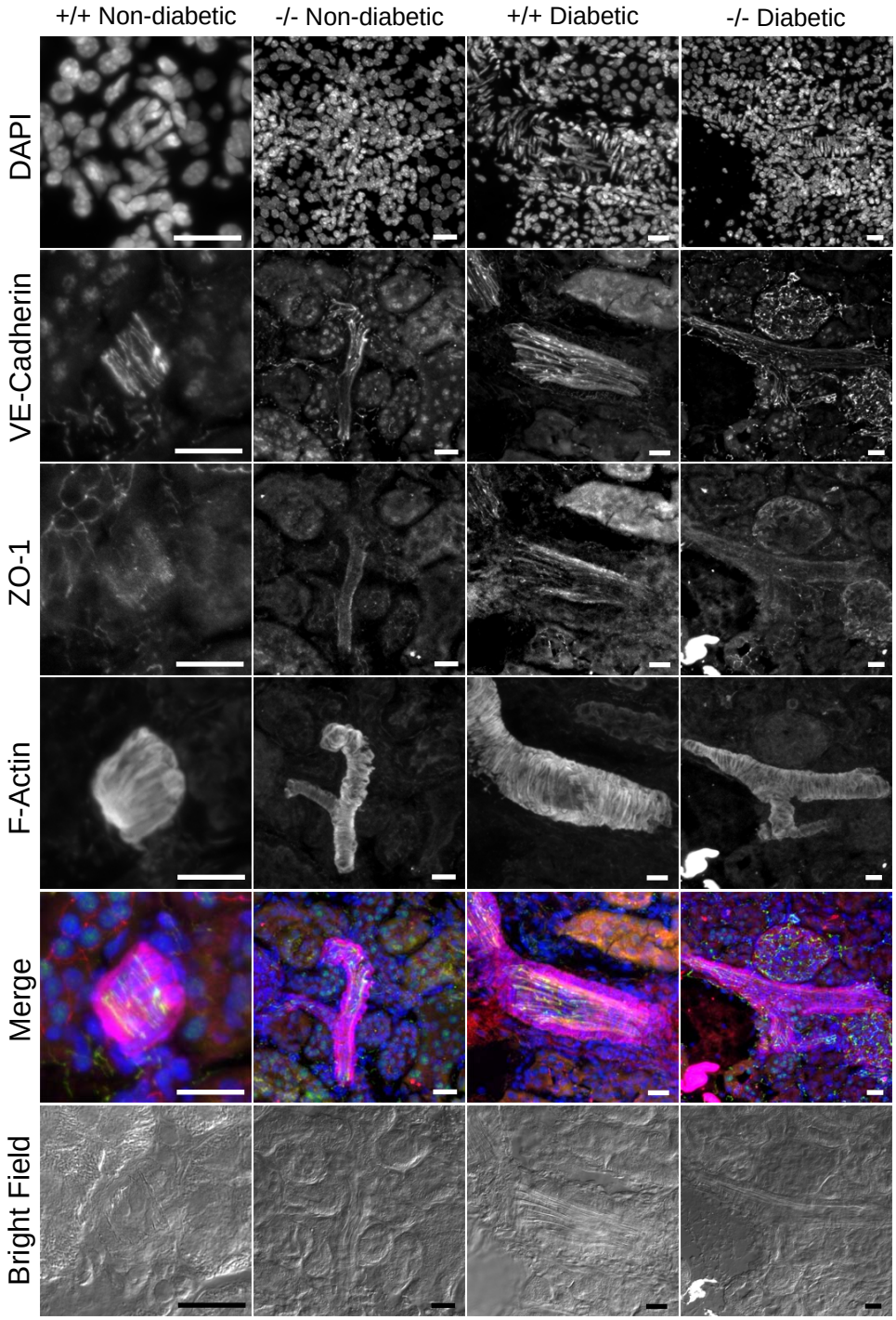

Fig 5.14: Assessment of kidney vessel junctional protein localisation.
Kidney tissue was sectioned, fixed and stained for analysis by confocal microscopy as detailed in the materials and methods. Vessels were then imaged, identified by the intense F-actin stain, with a representative from each group of mice shown here, sections were stained using antibodies and reagents: DAPI (1:500), Anti-VE-cadherin (1:50), Anti-ZO-1 (1:300), Phalloidin-633 (1:60), Anti-Goat-Alexafluor-488 (1:1000), Anti-Rabbit-Alexafluor-568 (1:1000). Scale bar = 20μm

annexin 2 expression levels western blotting was performed on samples from eye, brain microvasculature and kidney of AnxA2$^{+/+}$ non-diabetic and diabetic mice. Levels of annexin 2 were quantified by densitometry and normalised to tubulin. Annexin 2 up-regulation was evident in most tissues, particularly the brain microvasculature, increasing three fold over control (Figure 5.15). Levels of annexin 2 in the eye were approximately double those of the control, whereas kidney annexin 2 was elevated 1.7 fold. Due to the preliminary nature of the experiment and hence low sample numbers, significant differences could only be assigned to the kidney ($p<0.05$), however it can be seen from both the brain vasculature and eye data that greater sample numbers may have yielded similarly significant results.

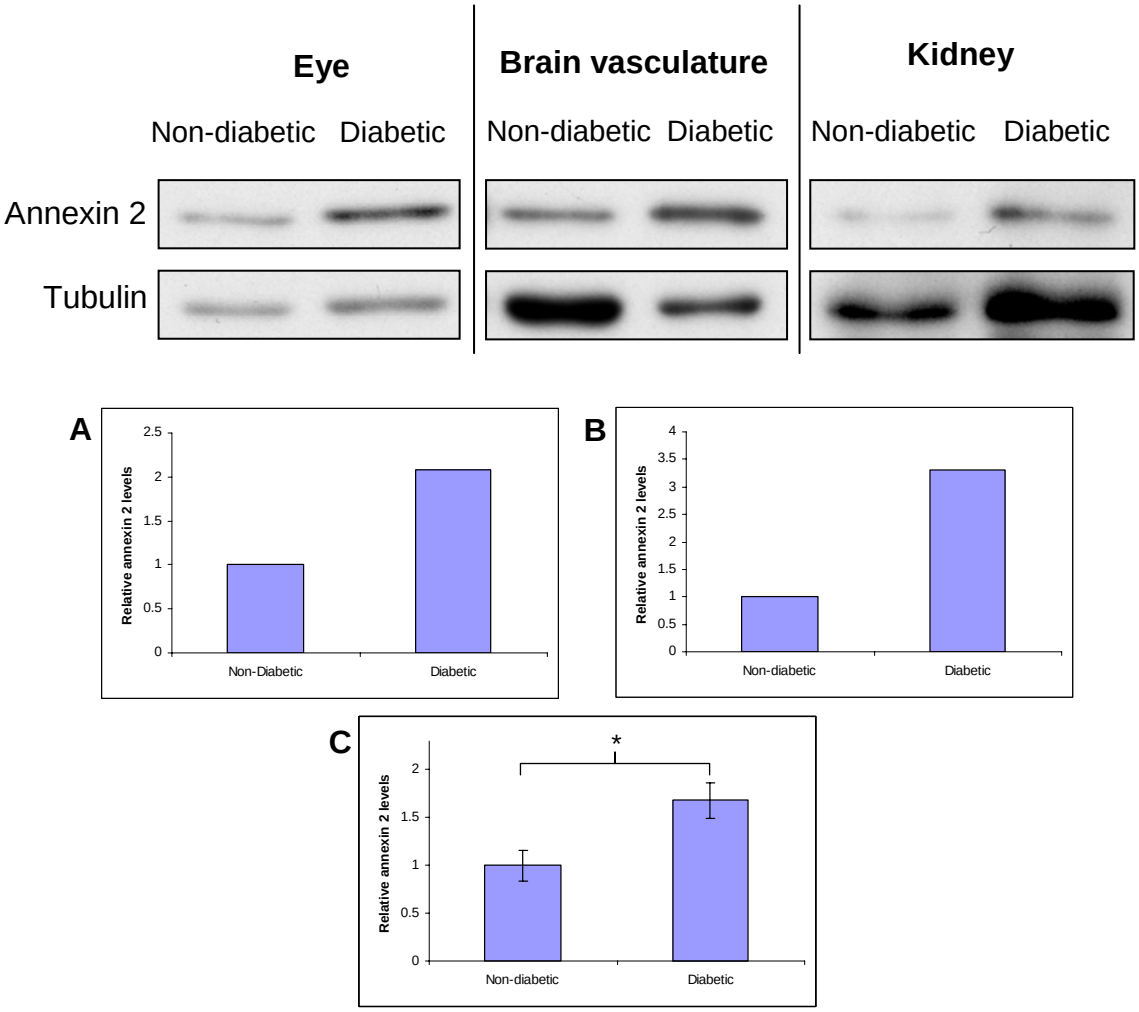

Fig 5.15: Annexin 2 is up-regulated in the diabetic eye, brain vasculature and kidney.
Protein samples were isolated from the eye (n=1, graph A), brain vasculature (n=2, graph B) and kidney (n=3, graph C) of AnxA2$^{+/+}$ non-diabetic and diabetic animals as described in the materials and methods. Lysates were then western blotted for annexin 2 and tubulin as shown above and densitometry conducted using Image-J to give levels of annexin 2 normalised to tubulin. Error bars denote ±sd. Antibodies: Anti-annexin-2 (1:1000; BD Biosciences), Anti-tubulin (1:10) and Anti-mouse-HRP (1:5000). Bands resolved using ECL. *=$p<0.05$

Chapter 6 Discussion

Chapter 6 Discussion

Annexin 2 has been shown to be involved in many different cellular processes, some of which are implicated in the endothelial dysfunction that occurs in diabetes. To assess whether annexin 2 was involved in the endothelial cell response to hyperglycaemia, cells were cultured in a range of conditions, namely euglycaemia, 15mM glucose, 25mM glucose and +20mM mannitol for one week, in order to permit comparision with other studies that examined endothelial cell function in this context[406,407,408]. Levels and distribution of annexin 2 were then examined, followed by assessment of cells lacking annexin 2, in order to explore a hypothetical role for annexin 2 in the endothelial dysfunction seen in diabetes.

6.1 Assessment of levels of annexin 2 using *in vitro* hyperglycaemia

Annexin 2 levels have been reported to fluctuate in various cellular processes, as has been illustrated in studies of annexin 2 during the cell cycle[409]. It has been previously demonstrated by Lei *et al* that the levels of annexin 2 found on the surface of endothelial cells increase in response to hyperglycaemic conditions[378]. This could not be confirmed in our study, since persistent tubulin contamination of the extracellular samples was indicative of intracellular contamination. Although the assessment of extracellular annexin 2 was problematic, its elution from the surface of the endothelial cells did allow for the accurate assessment of intracellular annexin 2 levels in response to *in vitro* hyperglycaemia. In both HUVEC and hCMEC/D3 endothelial cells, culture in hyperglycaemia for 1 week failed to alter the intracellular levels of annexin 2, with GPNT cells cultured for up to 9 weeks demonstrating this effect was also consistent at later time points. Since hyperglycaemia has been shown to increase the levels of annexin 2 on the surface of the endothelium through increased binding to heat shock protein 90α[378], one might postulate that although the protein levels of annexin 2 do not change in response to hyperglycaemia, there may nevertheless be effects on the transcriptional regulation of the annexin 2 gene.

6.2 Examination of the alterations in distribution of annexin 2 in response to hyperglycaemia

Since the levels of annexin 2 remained stable in response to hyperglycaemia, changes in it's distribution were examined using confocal microscopy. Proteins known to be involved in the progression of diabetes in endothelial cells, namely GLUT-1[410], ZO-1[411], VE-cadherin[412] and F-actin[413] were also examined, such that any change in their distribution might be linked to changes in annexin 2.

In this study we identified subtle changes in the perinuclear levels of GLUT-1 when HUVEC were exposed to *in vitro* hyperglycaemia, with a slightly increased signal in cells treated with 25mM glucose, whilst those treated with 15mM glucose appeared to exhibit a decrease in perinuclear GLUT-1. The possibility of changes in the perinuclear distribution of GLUT-1 in response to hyperglycaemia add to an already controversial subject since there is still some debate as to whether GLUT-1 levels are increased or decreased in response to hyperglycaemia[388,389], and similarly whether there is an increased or decreased level of glucose transport[414,415], the controversy arising at least in part due to a lack of similarity between studies. Since GLUT-1 is known to shuttle to membrane compartments to adjust the glucose permeability of the cell[416,417] it may not be unreasonable to assume changes in its distribution could explain some of the disparities in results, especially since diabetes is sufficient to alter elements of the endocytosis pathway[418], and the extent of those changes is dependent upon length of hyperglycaemic insult and endothelial cell type examined.

The results of this study also showed that the distribution and organisation of F-actin was altered in response to hyperglycaemia. Actin is a highly dynamic protein altering in response to various stimuli[419]. In times of cellular stress actin 'stress fibers' are formed[420] and hence it is not unsurprising to see them formed here in a hyperglycaemic dose-dependent manner. Whether in this case they formed as a direct consequence of hyperglycaemia alone is questionable, since stress fibers also formed in cells treated with increased mannitol, and hence may be due to increased osmotic stress. Changes in the distribution of actin are of potential interest regarding annexin 2, since the two proteins are closely linked as detailed in the Introduction. As annexin 2 is known to function as an actin capping protein[323], the loss of annexin 2 leads to the formation of stress fibers[323], and annexin 2 is one of the first proteins to become glycated[375] it would be interesting to determine whether glycation interferes with annexin 2 function in a manner that leads to the formation of stress fibers.

The other proteins examined, namely VE-cadherin, ZO-1 and annexin 2, remained constant, in that *in vitro* culture in hyperglycaemia was insufficient to cause any loss of junctional protein localisation, or alter their distribution. Whilst the

maintenance of ZO-1 at the cell junctions in response to hyperglycaemia is consistent with other studies[421], data from the literature indicate that significant changes in VE-cadherin distribution may have been more readily seen at an earlier time point, since although discontinuous staining of VE-cadherin was evident after 2 hours in HUVEC treated with 25mM glucose, the significance of this result was lost after 4 days of culture[422]. No comparison of the result obtained for annexin 2 can be made, since the response of annexin 2 to hyperglycaemia has not previously been examined in this way. However, the interpretation of annexin 2 distribution should be undertaken with care since the antibody used is directed against the N-terminal region of the protein, near the site of its interaction with S100A10. Thus, staining of annexin 2 in these figures is probably indicative of monomeric annexin 2 only, since the tetrameric annexin 2-S100A10 complex is inaccessible to the antibody. It is also the case that most of the annexin 2 stained is in the cytosol, which may mask subtle enrichments to cellular organelles. For this reason, changes in the distribution of annexin 2 were next examined using biochemical techniques that would allow the examination of the total pool of annexin 2, and hence may reveal changes not detectable by confocal microscopy.

In these experiments, we utilised one of the key properties of annexin 2 in its ability to bind membranes. A crude method of isolating membrane fractions is to use detergent to obtain detergent-soluble and insoluble fractions[423]. Although this method has been shown to be effective in other studies, in our hands annexin 2 could not be efficiently fractionated. An alternative protocol utilising Percoll™ similarly failed to provide results that could be accurately interpreted, but did provide better fractionation than the detergent-based method. The most satisfactory result were obtained using a sucrose density gradient approach, which has been shown to be effective in other work examining the effects of diabetes, more specifically oxidative stress and its role in translocating annexin 2 to the endoplasmic reticulum in Hela cells[424]. Using this method it was observed that the actions of hyperglycaemia were sufficient to move the peak of annexin 2 to a denser fraction, in a dose-dependent manner. This result was confirmed by reference to the distribution of transferrin receptor, a marker of early endosomes, which remained in the same fractions regardless of the culture conditions, illustrating that the effect on annexin 2 was not due to variations in the sucrose gradient.

The question arising from this experiment concerns to which membranous compartment of the cell is annexin 2 moving, and what is inducing this effect. Although the stimulus for this effect is undoubtedly incubation in hyperglycaemia, the actions of hyperglycaemia can have various effects on the endothelial cell, as outlined in the

Introduction. Pertaining to annexin 2, in the literature the actions of diabetes have been reported to potentially alter the structure or function of annexin 2 through glutathionylation and glycation. More specifically, *in vitro* oxidative stress has been shown to induce the glutathionylation of both the monomeric and tetrameric forms of annexin 2 at cysteines 8 and 132[425,426]. The glycation of annexin 2 was demonstrated *in vivo* using streptozotocin induced diabetic rats, whereby after one month of diabetes, membranous compartments of the lung vasculature were shown to contain glycated annexin 2, along with glycated forms of components of the the actin cytoskeleton including the p34 subunit of the Arp2/3 complex, Ras suppressor protein-1 and actin[375]. Since these events are described as early events in diabetes it is possible that either, or both of these processes may have provided stimulus for the effect seen in the sucrose gradient. Deliberating next upon how these modifications may have caused the movement of annexin 2 to other compartments of the cell, glutathionylation has been shown to alter the function of annexin 2, reducing its capacity to bind membranes by up to around 70%[425], whilst glycation has been shown *in vitro* to reduce the functionality of other proteins such as collagen, delaying wound healing[427]. If it is then further considered that work in this thesis has demonstrated that the levels of annexin 2 protein are not increased by 1 week culture in hyperglycaemia, with potentially more actin stress fibers formed in HUVEC exposed to 25mM glucose, it could be postulated that the movement of annexin 2 to alterative compartments of the cell could be indicative of inactivation of the protein and possibly degredation, especially since lysosomes are more dense than that of early endosomes[428]. Alternatively, since annexin 2 translocation to alternative membrane structures has been demonstrated[429] in reaction to stress responses, such as hyperosmolarity, phorbol ester or pervanadate and high density compartments of the cell, with as of yet unknown function, containing caveolin have been identified[430], it is not unreasonable to hypothesise that annexin 2 may be translocating to serve a functional requirement.

6.3 *In vitro* hyperglycaemia in AnxA2$^{-/-}$ endothelial cells

Roles for annexin 2 in the progression of diabetes have been postulated both by our group and others, potentially identifying diabetes as an annexinopathy, and are described in detail in the Introduction. Briefly, the hypotheses focus around the increased presence of annexin 2 on the surface of endothelial cells in response to hyperglycaemia due to its increased binding to heat shock protein 90α[378]. Interestingly,

whether this translocation serves to induce more or less plasmin formation, for which annexin 2 has an established role[359,64], is disputed[376,378]. The major difference between the two studies was the addition of insulin to the experimental procedure[431], and since annexin 2 has been shown to be phosphorylated by the insulin receptor[379,380], this suggests that phosphorylation may decrease the potential for annexin 2 to aid plasmin formation, especially since other modifications close to the site where annexin 2 is phosphorylated can reduce its capacity to bind t-PA[359,383,382]. In both hypotheses the contribution of annexin 2 to the progression of diabetes is centred around its ability to aid plasmin formation, altering the fibrinolytic balance of the endothelium.

Since earlier work in this thesis had suggested that the actions of hyperglycaemia may reduce the availability or change the function of annexin 2, we next investigated the consequences of diabetes on endothelial cells lacking annexin 2. For this purpose we used the annexin 2 knockout mouse[361], in which two exons have been replaced with a neomycin resistance gene. The annexin 2 knockout mouse ($AnxA2^{-/-}$) has been used previously to demonstrate the role of annexin 2 in fibrin generation, neoangiogenesis, haematopoietic stem cell homing and stabilisation of its binding partner S100A10. More specifically, mice lacking annexin 2 have been shown to accumulate fibrin in the microvasculature, demonstrate incomplete clearance of arterial thrombi induced by injury and have reduced t-PA induced plasmin formation[361]. In addition, isolated endothelial cells or aortic ring explants show reduced migration through Matrigel, demonstrating that the reduction in plasmin formation has functional consequences *in vitro*[361]. Further to this, the authors also utilised two *in vivo* models of neovascularisation, namely FGF-stimulated cornea and oxygen induced retinopathy, showing that $AnxA2^{-/-}$ mice exhibit less neoangiogenesis, supporting the idea of a role for annexin 2 in the degradation of the extracellular matrix to permit neovascularisation *in vivo*. The same mice have also been used in three other studies, in a publication by Jung *et al* it was demonstrated that the $AnxA2^{-/-}$ mice have fewer haematopoietic stem cells in their bone marrow, which in addition to various treatments of wild-type mice and cell cultures with blocking peptides, RNA interference and antibody treatments directed against annexin 2, implicates annexin 2 in the homing of haematopoietic stem cells to the bone marrow[432]. Second, loss of annexin 2 in mice has been shown to reduce the levels of its binding partner S100A10, since in the absence of annexin 2 the ubiquitination site of S100A10 is unprotected leading to its constitutive degradation [363]. Finaly, annexin 2 has been shown to have a role in the activation of Src and FAK in the

circadian phagocytosis of photoreceptor outer segments by retinal pigment epithelial cells[342].

As part of our investigations using the AnxA2$^{-/-}$ mouse, isolation of endothelial cells from the brain microvasculature demonstrated that the loss of functional annexin 2 protein was insufficient to disrupt VE-cadherin localisation from the membrane of the cells, either in the presence or absence of hyperglycaemia. This may seem surprising since annexin 2 has been implicated in the stabilisation and formation of cell junctions of both vascular and non-vascular cells through interactions with proteins such as Rac1 in epithelial cells[343], in addition to targeting of E-cadherin to the junction of epithelial cells as a result of calcium depletion and subsequent restoration[344]. Indeed, annexin 2 has also been shown to be important for VE-cadherin stabilisation since the loss of annexin 2 from HUVEC cells has been shown to be sufficient to cause mis-localisation of the protein and hence de-stabilisation of the adherens junction[349]. With the added parameter of hyperglycaemic insult that has been shown to be detrimental to junctional stability[433], the results obtained herein are unexpected given the current literature. There are potential reasons for this discrepancy, not least that the cells isolated as part of this study are derived from the microvascular brain endothelium, and the vascular cells used in the study of annexin 2 function on VE-cadherin stabilisation were conducted in HUVECs. As outlined in the Introduction, endothelial cells are adjusted to the environment in which they find themselves[28,29]. Since the junctional integrity of the endothelial cells that comprise the blood brain barrier is particularly strong, mechanisms may be in place to protect these junctions from changes in either glycaemia or protein composition. Indeed, studies have demonstrated both in diabetic patients[434] and animal models of diabetes[435] that the integrity of the blood brain barrier is preserved, and no indication of vascular leakage, examined by the presence of albumin, sucrose or IgG in the brain tissue, can be found after extended periods of diabetes. However, contrary evidence has also been published similarly both in diabetic patients[436], and animal models of diabetes[437], illustrating that the blood brain barrier is susceptible to permeability changes as a consequence of diabetes. Thus, the actions of diabetes on the blood brain barrier are still under debate.

Another potential reason for the apparent inconsistency of the results with the published literature is that although genetic knockout mouse models are a versatile and powerful tool for scientific research, they do have their own distinct disadvantages[438], amoung which is the potential for adaptation to the loss of the given protein of

interest[439,440], in this case annexin 2. Hence, it may well be possible that other annexins are up-regulated or their activity increased to compensate for the loss of annexin 2.

In summary, through *in vitro* studies we have demonstrated that the levels of annexin 2 protein are not altered in response to hyperglycaemia, but it's distribution may be altered, with regard to subcellular compartmentalisation. In addition to this, it was found that the loss of annexin 2 was not sufficient to accentuate any effect hyperglycaemia may have on junctional integrity, but that this may have been a consequence of the type of endothelial cell used.

Although *in vitro* studies of diseases such as diabetes provide a valuable insight into the mechanics of disease progression, many of these conditions are multifactoral in nature. Since diabetes is no exception, and the resulting hyperglycaemia affects many different cell types changing multiple cellular processes, as outlined in the Introduction, a true representation of the contribution of annexin 2 to the actions of diabetes can only be assessed using *in vivo* model of disease. Thus, using both AnxA2$^{+/+}$ and AnxA2$^{-/-}$ mice a chemically induced models of diabetes was instigated, allowing the contribution of annexin 2 function to be addressed in the setting of hyperglycaemia, and the plethora of other factors involved in diabetic endothelial dysfunction.

6.4 Streptozotocin induced diabetes in AnxA2$^{-/-}$ mice

In vivo models of diabetes have been used for many investigations employing both genetic and chemical induction, as outlined in a review by Rees & Alcolado[441]. Streptozotocin is a drug widely used to induce diabetes in rodents, with more studies in rats, than mice. Since the streptozotocin model of diabetes is well established, more recent papers have examined the effects, both *in vivo* and *in vitro*, of exogenous reagents, genetic alteration or protein changes[390,391,392,393,394,395,396,442].

Weight loss, or reduced weight gain, is a symptom of uncontrolled diabetes. In our study both AnxA2$^{+/+}$ and AnxA2$^{-/-}$ mice treated with streptozotocin displayed reduced weight gain and hyperglycaemia which were significantly different than their control littermates. The observation of reduced weight gain is consistent with other studies that have utilised streptozotocin, demonstrating that diabetic mice either have a reduced weight to that of their controls at the point of sampling[443,444], or that diabetic mice fail to gain weight over the period of time they were examined[445]. Hence, our observations confirm that diabetes has been successfully induced in these animals. In addition since no detectable difference in weight gain / loss was observed between the

AnxA2$^{-/-}$ and AnxA2$^{+/+}$ mice regardless of diabetes, it can therefore be concluded that the loss of annexin 2 is not sufficient to change either weight gain as part of normal physiology, or exacerbate the lack of weight gain / weight loss incurred by the actions of diabetes.

Diabetic mice were observed to have blood glucose levels significantly higher than their control littermates at two weeks post streptozotocin treatment. The levels of hyperglycaemia achieved in this study for both AnxA2$^{+/+}$ and AnxA2$^{-/-}$ mice were somewhat higher than those quoted in the literature for c57bl/6 mice treated with the low-dose streptozotocin protocol at approximately 300mg/dl[446,447,448], potentially due to an inadequate response to fasting; as will be elaborated upon later. An alternative explanation acknowledges that differences in the strain of mouse used can produce varying levels of hyperglycaemia[449,450], with slight differences even detected between very closely related c57bl/6J and c57bl/6KsJ[451]. A more recent study examining the differences in strain regarding the progression of diabetic nephropathy in response to streptozotocin demonstrated this phenomenon effect in five different strains of mice namely, MRL/Mp, BALB/c, 129/EvSv, c57bl/6 and Dba/2[452].

However, since the level of hyperglycaemia achieved as part of this study was higher than that quoted in the literature for the strain used, and the data regarding reduced weight gain were consistent with the model, it was concluded that we had successfully induced diabetes in these mice. Interestingly the time course of glucose increase was similar between AnxA2$^{+/+}$ and AnxA2$^{-/-}$ diabetic mice, indicating that the loss of annexin 2 does not affect the onset of chemically induced diabetes.

Upon analysis of the data in non-diabetic animals it was observed that fasting for 4 hours increased blood glucose relative to the non-fasted state. This is both the opposite of the expected result and not in accordance with the literature, since studies have shown fasting of non-diabetic mice to yield an approximately 50mg/dl reduction in their blood glucose level[453,454,455]. However, since mice were placed into fresh cages at the point of fasting, and this was a consistent result across the 16 week study, it brings into question the murine response to food withdrawal and blood glucose sampling. In the process of obtaining blood glucose levels mice were restrained, such that the tail vein could be accessed easily. However, restraint has been shown to increase the levels of blood glucose in mice[456,457]; with c57bl/6 mice particularly prone to hyperglycaemic responses to restraint induced stress[458]. Other studies have also shown that the levels of blood glucose increase in mice when active[459], and since mice post restraint were active

for extended periods of time, this could have contributed to the observed hyperglycaemic response to fasting.

Interestingly, regardless of fasting, non-diabetic AnxA2$^{-/-}$ mice had a significantly lower level of blood glucose than their wild-type counterparts, indicating that the loss of annexin 2 results in abnormal glucose handling. Many different knockout mouse models have reported similar results by alteration of various aspects of the glucose homeostasis system. Alteration to the insulin signalling pathway, either through increased insulin transcription by reduction of Smad3 levels[460], enhancement of insulin signalling through depletion of SOCS7[461], or increased sensitivity to insulin through deletion of the p85α regulatory subunit of PI3K[462], have been shown to induce hypoglycaemia in mice. In addition, disruption of the thioredoxin pathway[463], or reduction of the ability of the pancreas to release insulin or glucagon[462] can also induce hypoglycaemia. As well as these alterations to established constituents of the glucose homeostasis pathways, another annexin, namely annexin 7 has been implicated in these processes. In a study by Srivastava *et al* the β-islet cells of the pancreas were observed to express different levels of proteins in the fed or fasted state, however, upon loss of annexin 7 these cells were no longer able to adapt to the change in environment and gene expression remained the same regardless of whether they were fed or fasted[464]. The mice were also shown to have increased glucose clearance after glucose tolerance testing, with their peripheral tissues hyper-responsive to insulin. However, no significant hypoglycaemia was seen when control mice were compared to the annexin 7 knockouts.

Since the AnxA2$^{-/-}$ mouse has not been used previously to investigate glucose homeostasis, this is the first report of hypoglycaemia in these mice. Potential explanations for this observation can be inferred from the literature, focusing on the known responses of annexin 2 to insulin signalling. Annexin 2 has been shown to be phosphorylated by the actions of the insulin receptor, and to mediate cellular alterations to the actin cytoskeleton dependent upon phosphorylation, that can be inhibited upon knockdown or neutralisation of the protein[379,380]. Signalling events mediated by the insulin receptor occur both when the insulin receptor is in the membrane, and upon endocytosis[465]. If either the endocytosis of the receptor is inhibited, or the signalling of the endocytosed receptor is enhanced, hypoglycaemia can ensue via increased glucose uptake, increased glycogen synthesis and an increase in its downstream signalling pathways[466,467,468]. Since annexin 2 has been shown to be involved in endocytosis[332], particularly in response to phosphorylation[336], it would not be unreasonable to

hypothesise that loss of annexin 2 would be sufficient to alter the endocytic pathway, such that the correct sorting of the insulin receptor is affected, and abnormal signalling events occur. Another potential explanation for the apparent hypoglycaemia of AnxA2$^{-/-}$ mice is closely linked to the insulin signalling pathway, and centres around GLUT-4. When stimulated by insulin GLUT-4 moves to the plasma membrane, where it mediates the uptake of glucose into the cell for its utilisation[469]. Recently a pool of insulin-unresponsive GLUT-4 was identified in muscle, in which annexin 2 was enriched[470]. The authors of the paper hypothesised that this new pool of GLUT-4 was continuously circulating between the plasma membrane and the new intracellular pool, the rate of which might be influenced by insulin signalling. Since, as outlined above, annexin 2 can mediate the effects of the insulin signalling pathway, and has established roles in endocytosis, it could be hypothesised that the presence of annexin 2 is critical for the correct functioning of this insulin-insensitive pool of GLUT-4. If ablation or reduction of annexin 2 protein resulted in more of this insulin-insensitive pool of GLUT-4 in the plasma membrane, the peripheral utilisation of glucose would be increased, and hence hypoglycaemia would ensue.

Upon the initiation of diabetes in these animals, fasted diabetic AnxA2$^{+/+}$ and AnxA2$^{-/-}$ mice exhibited similar levels of hyperglycaemia ranging from 500-600mg/dl. Interestingly, when the same mice are not fasted prior to their blood glucose measurement a different trend becomes apparent, with AnxA2$^{-/-}$ diabetic mice having a significantly higher blood glucose level than AnxA2$^{+/+}$ counterparts. Both of these observations conflict with the previous observation that non-diabetic AnxA2$^{-/-}$ mice have a lower basal blood glucose level. However, if it is considered that in non-diabetic mice the increase in the insulin signalling pathway may be a compensatory mechanism to an increase in endogenous glucose production, through increased gluconeogensis, the subsequent hyperglycaemia of these AnxA2$^{-/-}$ mice upon the actions of diabetes, and hence ablation or reduction of insulin signalling, could be explained. Although an increase in the rate of gluconeogenesis in various knockout animal models has been reported[471,472,473], no such association of annexin 2, or any other annexin, could be attributable to the mechanisms that influence the rate of gluconeogenesis, reviewed in[474], and hence this rationalisation for the effect seen in diabetic AnxA2$^{-/-}$ mice shall remain a postulation.

More relevant to the previous data, other knockout mouse models have also reported that higher levels of hyperglycaemia can be induced upon initiation of streptozotocin induced diabetes, due to a dysfunction in the insulin signalling pathway

prior to the onset of diabetes utilising mice with a liver-specific knockout of IGF-1[475], and alterations to the ability of the pancreatic islets to secrete insulin in mice lacking Group VIA phospholipase A(2)[476]. As outlined above AnxA2$^{-/-}$ non-diabetic mice show hypoglycaemia indicative of a dysfunctional regulation of the insulin signalling pathway, and hence this may explain the differences observed in AnxA2$^{-/-}$ mice upon the initiation of diabetes.

A further interesting observation that can be drawn from the diabetic blood glucose data, is the exaggerated reduction of blood glucose levels in the AnxA2$^{-/-}$ diabetic mice in response to fasting. As stated previously non-diabetic mice examined as part of this study increased their blood glucose levels in response to fasting, and although this is not consistent with published literature regarding fasting of mice, it may have been produced by a stress response. However, upon examination of the diabetic mice, whereas AnxA2$^{+/+}$ mice maintained their blood glucose concentration in response to fasting, AnxA2$^{-/-}$ diabetic mice exhibited a significant reduction. Potential explanations for this effect once more return to the insulin signalling pathway, and the defects the absence of annexin 2 may cause. In models of disruption of the insulin signalling pathway, previously outlined above, in addition to the effects on insulin signalling, an increase in peripheral glucose uptake was observed[112,460,461,462], examined through glucose tolerance testing. In these experiments, animals with increased peripheral glucose uptake reduced their glucose levels quicker in response to a bolus injection of glucose than that of their control littermates, an effect that is analogous to the increased reduction of blood glucose levels in AnxA2$^{-/-}$ diabetic mice post fasting. Since in the absence of annexin 2 non-diabetic mice exhibit hypoglycaemia, potentially through increased peripheral glucose uptake, it would not be unreasonable to hypothesise that the same would be true when these animals were diabetic, and therefore the data presented here in these mice upon the action of diabetes may provide further evidence in support of the hypothesis.

In summary, although the loss of annexin 2 does not affect the onset of diabetes in the streptozotocin model, differences can be found in glucose handling. AnxA2$^{-/-}$ non-diabetic mice exhibit hypoglycaemia potentially either due to dysregulation of the insulin signalling pathway, mislocalisation of GLUT-4, increased glucose uptake in the peripheral tissues or a combination of two or more of these. An increase in the rate of glucose uptake in the peripheral tissues is likely since in the AnxA2$^{-/-}$ diabetic animals glucose levels reduced more drastically in response to fasting.

6.5 Diabetic retinopathy and AnxA2$^{-/-}$ diabetic mice

Having successfully established a model of diabetes in the AnxA2$^{-/-}$ mice we set about examining whether the loss of annexin 2 could have any detrimental effects on the microvascular disease evident in diabetes, starting with diabetic retinopathy. Fluorescein angiograms taken at 16 weeks post diabetes induction demonstrated that there were no differences between AnxA2$^{-/-}$ and AnxA2$^{+/+}$ diabetic animals, and that diabetic animals had normal retinal vasculature. Other studies that have examined diabetic retinopathy by fluroescein angiography typically examine later time points[205] since it can take up to a year for vascular leakage as a result of diabetic retinopathy to present. A much earlier time point was taken in this study since diabetes in our animals proved lethal post 16-18 weeks of diabetes in the majority of cases. This level of mortality is in contrast to other studies that have utilised the streptozotocin induced diabetes model to examine mice over a long period of time[477,478]. However, in these studies streptozotocin treated animals underwent routine insulin treatment linked to their weight gain / loss to proling survival, so it is not surprising that differences are apparent.

Since our study was not long enough to examine diabetic retinopathy by fluorescein angiography, earlier symptoms of the disease were examined. One of the initial events to occur in the retina in response to hyperglycaemia is the loss of pericytes[479]. In the absence of specific antibodies for pericyte cells, the retina could not be efficiently examined using fluorescence microscopy, and hence pericyte dropout was examined using a conventional histological method. To achieve this PAS staining with a haematoxylin counterstain of trypin digested retina revealed the nuclei present in the vasculature, which could then be identified as either endothelial cells or pericytes. Various publications have used this method to study pericyte dropout in the diabetic retina in both rats[397,400,401] and dogs[398,399]. In our study, whilst significant differences in the pericyte to endothelial cell ratio were detected between the control and diabetic mice of either genotype, indicating changes indicative of retinopathy had occurred in these animals, differences were not apparent between AnxA2$^{+/+}$ or AnxA2$^{-/-}$ mice.

Since the investigation of pericyte dropout did not reveal any significant differences between that of AnxA2$^{+/+}$ and AnxA2$^{-/-}$ mice, an earlier manifestation of diabetic retinopathy was assessed. As detailed in the introduction, the metabolically stressed retina up-regulates GFAP in Müller cells, hence we examined GFAP in the retinas of the AnxA2$^{-/-}$ diabetic mice. No significant up-regulation of GFAP in the inner

plexiform layer was detected in either the AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic or diabetic mice. Although these data are contrary to those published for rat, in which a substantial up-regulation of GFAP can be seen after streptozotocin treatment for 10[480] and 24[481] weeks, or incubation of retinal explants to glycated-BSA for 4 days[482], it is consistent with publications in mice that demonstrate no GFAP up-regulation can be detected in mice at various time points ranging from 2 weeks to 22 months[483,478].

In the absence of significant differences between AnxA2$^{+/+}$ and AnxA2$^{-/-}$ diabetic animals upon examination of the retina, both by pan-retinal examination for pericyte dropout, and specific examination of metabolic stress and hence GFAP up-regulation, cross-sections of retina were examined using conventional histology to see if any abnormalities could be detected. Upon analysis of the data no abnormalities in any of the retinas were detected. Similar studies have also used this technique to quantify retinal cell death in streptozotocin induced diabetic rats, observed as a reduction in retinal thickness[402]. The data obtained as part of this study did show some changes in retinal thickness, but these were evenly distributed across all the different mice, indicating that the changes were due to variations in sectioning and not the result of cell death.

Finally, since diabetic retinopathy is thought to affect the vessel junctions leading to leakage either through the actions of VEGF[224,484,485], MMPs[486] or inflammatory factors[487,488], as detailed in the Introduction, we next investigated whether *in vivo* diabetes could influence the distribution of cell junction proteins in the endothelium. To achieve this, endothelial cells were isolated in a similar manner to the previous data examining the short term effects on AnxA2$^{-/-}$ endothelial cells exposed to hyperglycaemia *in vitro*. In contrast to the previous data, endothelial cells from diabetic mice had been exposed to *in vivo* hyperglycaemia, and hence were more physiologically relevant than normal endothelial cells exposed to *in vitro* hyperglycaemia for one week. Upon examination of these cells it was seen that the yield from diabetic animals was much lower than from non-diabetic animals, suggesting that exposure to diabetes for 16 weeks had altered the cells' ability to survive the isolation procedure or attach to the surface of the culture dish. Similar to the previous experiment, staining of VE-cadherin was then conducted, revealing areas of discontinuous staining in AnxA2$^{-/-}$ diabetic endothelial cells which were not evident in either the control animals or their diabetic AnxA2$^{+/+}$ counterparts. This would indicate that exposure of cells lacking annexin 2 to *in vivo* hyperglycaemia for 16 weeks is sufficient to alter their ability to form and maintain VE-cadherin adherens junctions. Reasons for this effect are difficult to dissect

from these data alone, but would likely involve hyperglycaemic memory; the process by which cells exposed to hyperglycaemia are irreversibly damaged, leading to an accumulative effect even during periods of euglycaemia[489,490,491]. If it is assumed that the cells would have incurred a relatively large insult over the period of time exposed to diabetes *in vivo* it could be hypothesised that upon loss of annexin 2, endothelial cells are less able to cope with the cellular changes incurred when placed into an *in vitro* system where support from smooth muscle cells and systemic survival factors is absent or diminished.

When all the above examinations of diabetic retinopathy are combined it can be concluded that our study has shown that the loss of annexin 2 in diabetic mice is not sufficient to give differential disease presentation at 16 weeks post diabetes. However, what can be seen is that subtle changes may be taking place in endothelial cells, making them less conducive to isolation, and disrupting endothelial junctions when annexin 2 is absent. As mentioned previously other studies have utilised later time points to examine this disease, and any further studies would certainly need to examine later time points, in conjunction with insulin treatment to ensure that diabetic animals survive beyond 16-18 weeks post-induction.

Having demonstrated that the loss of annexin 2 has mild effects on the localisation of VE-cadherin, but no significant effects on the progression of diabetic retinopathy at 16 weeks, the effects one of the major cytokines implicated in diabetic retinopathy, namely VEGF[492], present at high levels in diabetic patients[224] was investigated using isolated AnxA2$^{-/-}$ endothelial cells. One of the effects of VEGF on endothelial cells in culture is the loss of VE-cadherin from cell junctions[493,494,495], hence we examined in our system whether the loss of annexin 2 influences the stability of VEGF induced VE-cadherin junction disruption. Through two independent studies we showed that VE-cadherin in AnxA2$^{-/-}$ endothelial cells was more sensitive to both the actions of VEGF, and non-specific shock through changing their media, illustrating that the loss of annexin 2 may alter the stability of VE-cadherin. Although this contrasts with our previous data showing that loss of annexin 2 was insufficient to alter the distribution of VE-cadherin in euglycaemia or hyperglycaemia *in vitro*, it is in agreement with the published literature regarding annexin 2 and VE-cadherin[349], and our data regarding the stability of VE-cadherin in endothelial cells isolated from AnxA2$^{-/-}$ diabetic mice. As outlined in the Introduction, in the work by Heyraud *et al* annexin 2 was observed to translocate to the membranes of endothelial cells upon confluence where it bound a complex containing VE-cadherin, the interaction of which

was independent of actin. Further to this, they demonstrated that disturbance of VE-cadherin localisation at cell junctions could be elicited using siRNA directed against annexin 2. The authors then went on to hypothesise that interactions of annexin 2 with SHP-2, illustrated in other work[347], and the potential for annexin 2 to act as a link between VE-cadherin and the actin cytoskeleton, may provide the mechanism by which annexin 2 stabilises VE-cadherin at junctions. Using this information, it could be hypothesised that the hyper-responsiveness of AnxA2$^{-/-}$ endothelial cells to VEGF is as a result of incomplete stabilisation of the VE-cadherin adherans junction, resulting in increased sensitivity to VEGF.

During the investigation of diabetic retinopathy in the AnxA2$^{-/-}$ mice, once it became evident that only mild alterations to the retina were apparent, the mice were examined for other potential symptoms of diabetic microvascular disease. One key observation was that the cages containing diabetic AnxA2$^{-/-}$ mice required cleaning more often than those containing AnxA2$^{+/+}$ diabetic mice. This indicated that there were some potential differences in the levels of urine excretion, and since the kidneys can be severely effected in diabetes, we next investigated whether AnxA2$^{-/-}$ diabetic mice displayed increased progression of diabetic nephropathy in comparison to their AnxA2$^{+/+}$ counterparts.

The initial observation of increased urine output was confirmed, indirectly, by examination of their water intake. AnxA2$^{-/-}$ diabetic animals were seen to consume significantly more water than AnxA2$^{+/+}$ mice. Since non-diabetic animals displayed similar levels of water consumption, this suggests that loss of annexin 2 alone is insufficient to change kidney function, and a physiological insult, in this case diabetes, is required for differences to become apparent. Other studies have also examined the level of water consumed by rats exhibiting diabetic nephropathy[496] and report similar increases. However, more commonly the effect of diabetes on polydipsia is examined by measurement of urine excreted rather than water consumed, an approach that also shows large increases in urine output in rats or mice using both genetic and streptozotocin based models of diabetes[497,498,499].

6.6 Diabetic nephropathy and AnxA2$^{-/-}$ diabetic mice

Having provided indirect evidence for changes in the kidney function of AnxA2$^{-/-}$ diabetic mice, a further symptom of diabetic nephropathy was assessed, namely microalbuminuria. The presence of increased albumin in the urine is indicative

of decreased kidney function, and a potential risk factor for the development of diabetic nephropathy[500]. In our study AnxA2$^{-/-}$ diabetic mice displayed a statistically significant increased presence of albumin in the urine in comparison to their AnxA2$^{+/+}$ diabetic counterparts, which in turn exhibited a negligible increase in albumin excretion relative to their to their non-diabetic littermates, and consistent with data reporting that the c57bl/6 strain of mice is particularly resistant to diabetic nephropathy[501,452]. Interestingly non-diabetic mice also showed differences in the excretion of albumin, with AnxA2$^{-/-}$ non-diabetic mice having slightly higher urinary albumin levels than non-diabetic AnxA2$^{+/+}$ mice. This would indicate, contrary to the data examining polydipsia, that loss of annexin 2 is sufficient to induce some small changes in kidney function, and that the actions of diabetes exacerbate these changes.

Combined, the previous two data sets illustrate that loss of annexin 2 is sufficient to accelerate the progression of the early symptoms of diabetic nephropathy, both through increased urine output; measured by water intake, and by albumin excretion. One question that cannot be addressed from these data alone is whether the increased urine output and albumin excretion are due to changes in the selectively permeable barrier of the glomerulus, or due to reduced re-absorption of water in the nephron. A large body of work examining annexin 2 function in junction formation and regulation of apical transport has been conducted in kidney epithelial cells. Thus, annexin 2 has been shown to be important for the formation and maintenance of cell junctions in MDCK cells through Rac1[343] and AHNAK[502] induced cell polarisation, and cholesterol dependent recruitment of E-cadherin to the periphery of the cell[344]. In addition annexin 2, as part of the heterotetrameric complex containing S100A10 on the surface of MDCK cells, has been proposed to bring opposing membranes together, and hence have a role in tight junction formation[355], whilst annexin 2 has been reported to determine cell confluence through orchestration of the cPLA2/S100A10 interaction[503]. A role for annexin 2 in apical transport in these cells has also been shown, since MDCK cells lacking annexin 2 have reduced delivery of vesicles containing sucrase isomaltase to the apical membrane. Additionally, the correct localisation and functioning of the TRPV5 and TRPV6 channels involved in calcium re-absorption, has been shown to involve annexin 2[504,505,506]. These data might suggest that the most likely source of dysfunction would be the re-absorptive capacity of the nephron, rather than the selectively permeable barrier of the glomerulus. Of particular interest are data indicating that annexin 2 is found in complex with the channel protein aquaporin-2[371], and is important in its translocation to the membrane[507], as well as its responses to stimuli such

as vasopressin[508]. Aquaporin-2 is critical in the regulation of body water homeostasis, and since functional changes in its ability to localise to the plasma membrane, illustrated by two point mutations, can lead to polydipsia[509], it may be hypothesised that loss of annexin 2 in diabetes is sufficient to increase water intake and excretion above that of wild type animals due to the mis-localisation of aquaporin-2.

Having shown that the loss of annexin 2 has the potential to alter kidney function during diabetes, and potentially during normal physiology, the gross morphology and detailed histology of the kidney were examined to see if any changes could be detected. Consistent with published literature in the rat, both $AnxA2^{+/+}$ and $AnxA2^{-/-}$ diabetic mice had significantly increased kidney weights[510,511], although no differences in kidney weight between $AnxA2^{+/+}$ and $AnxA2^{-/-}$ diabetic mice were detected. Upon histological examination of the kidneys $AnxA2^{+/+}$ diabetic animals showed changes consistent with the progression of diabetic nephropathy, such as increased tubule dilation, and loss of nephrons[512]. $AnxA2^{-/-}$ diabetic animals displayed similar changes, but on a much greater scale, which were readily observed in the H&E and PAS stained sections. Interestingly, $AnxA2^{-/-}$ non-diabetic animals displayed no detectable changes in comparison to $AnxA2^{+/+}$ non-diabetic animals, suggesting that although there may be subtle differences in kidney function due to the loss of annexin 2, these are not sufficient to induce any gross histological changes, and are more likely to reflect changes at the subcellular level.

As mentioned previously, depletion of annexin 2 in MDCK cells has been shown to reduce the localisation of AHNAK to the plasma membrane, known to be important not only for cell polarisation, but also the maintenance of cell thickness *in vitro*[502]. Analysis of epithelial cell thickness *in vivo* in our $AnxA2^{+/+}$ and $AnxA2^{-/-}$ diabetic mice revealed that although diabetic animals displayed reduced tubule cell thickness when compared to their non-diabetic control, no significant differences were detectable between the $AnxA2^{+/+}$ and $AnxA2^{-/-}$ diabetic animals. Although no significant differences were detected, the small changes in cell thickness suggest that the loss of annexin 2 may have the potential to reduce cell tubule thickness, *in vivo*, since in both non-diabetic and diabetic $AnxA2^{-/-}$ mice the mean values were slightly lower than in the $AnxA2^{+/+}$ mice, consistent with observations *in vitro* in other studies[502].

A further symptom of diabetic nephropathy examinable by histology is the degree of mesangial matrix expansion. In agreement with the literature diabetic mice had increased levels of mesangial matrix in the glomeruli[513], equivalent to published

data regarding diabetic nephropathy in c57bl/6 mice[501,452]. AnxA2$^{-/-}$ diabetic animals also showed a significantly higher level of mesangial matrix expansionthan their AnxA2$^{+/+}$ counterparts, with AnxA2$^{+/+}$ and AnxA2$^{-/-}$ non-diabetic animals having similar levels of mesangial matrix. This result shows that whilst the absence of annexin 2 is not sufficient to increase the level of mesangial matrix, in the presence of diabetes, those animals without annexin 2 show more expansion than the wild type mice. The role for annexin 2 in this context is more likely to centre on its established role in plasmin formation[64]. Since annexin 2 is present in the mesangial cells and phosphorylated by the actions of PKC[514], which is activated in diabetes (reviewed in[515]), it would not be unreasonable to assume that it may have some role in plasmin generation on the surface of these cells[331]. In addition, since the suppression of plasmin activity is one of the defining steps in the accumulation of extracellular matrix[516], further reduction in the potential of these cells to produce plasmin, through removal of annexin 2, would therefore lead to a higher production of extracellular matrix, which concurs with our data here.

Having demonstrated that the loss of annexin 2 has potential effects upon the development of diabetic nephropathy through the use of light microscopy, further detailed examination of kidney histology was conducted in a preliminary study using electron microscopy. Overall examination of the kidney tubule cells revealed that while there were no major ultrastructural differences between the AnxA2$^{+/+}$ and AnxA2$^{-/-}$ mice in either non-diabetic or diabetic samples, the actions of diabetes were sufficient to perturb tubular cell structure, reducing villi length and cellular thickness consistent with the results obtained using light microscopy. In addition to examination of the tubular cells of the kidney the general structure of the glomerulus was examined. As outlined in the Introduction, a large part of the early manifestations of diabetic nephropathy can be attributed to changes in podocytes[517], such as increased apoptosis and foot effacement. In the images taken of glomeruli from diabetic mice it was noted that AnxA2$^{-/-}$ diabetic mice appeared to have increased effacement of the podocytic foot processes in comparison to the AnxA2$^{+/+}$ diabetic mice, which may also suggest increased apoptosis of these cells. Quantification of basement membrane thickness was also examined both for tubule and glomerular capillary basement membranes. AnxA2$^{+/+}$ and AnxA2$^{-/-}$ diabetic animals displayed an increase in basement membrane thickness, consistent with published literature utilising streptozotocin-induced diabetes in rats[512] and mice[518], however no differences were detected between AnxA2$^{+/+}$ and AnxA2$^{-/-}$ diabetic animals. These data suggest that although the loss of annexin 2 is not sufficient to increase

basement membrane thickness, a potential role for the disruption of the interactions between podocytes and the basement membrane of the glomerular capillary may be evident. Although a role for annexin 2 in podocytes has not been reported, the fact that annexin 2 has an established role in actin dynamics[323], known to be important for the correct functioning of the podocyte[519,520], suggests that loss of annexin 2 may affect the function of these cells or their ability to adapt to a diabetic environment.

To further address the question of the contribution of annexin 2 to diabetic microvascular disease in the kidney, the junctional proteins VE-cadherin and ZO-1 were assessed in the glomeruli of diabetic mice using confocal microscopy. Previous publications regarding the expression of ZO-1 in diabetic glomeruli have indicated a reduced level of the protein, with removal from the junction and therefore increased cytosolic staining[273]. In both the diabetic $AnxA2^{+/+}$ and $AnxA2^{-/-}$ glomeruli no such changes in ZO-1 localisation were observed, although since the publication stated used electron microscopy, the level of detail obtained here may be insufficient. Examination of VE-cadherin in these glomeruli showed staining indicative of intact junctions in both $AnxA2^{+/+}$ and $AnxA2^{-/-}$ non-diabetic and diabetic mice, with F-actin staining similarly unaffected, also suggesting that these cell junctions had not been disrupted. Taken together there results indicate that changes to the epithelial cells of the nephron as a result of the loss of annexin 2 may be the primary cause of the differences detected between $AnxA2^{+/+}$ and $AnxA2^{-/-}$ diabetic mice.

Finally, at the beginning of this thesis we set about examining whether *in vitro* hyperglycaemia up-regulates the amount of annexin 2 found intracellularly, and concluded that it was not. In cases of renal failure or renal damage, up-regulation of annexin 2 has been reported[370,521], and since differences between *in vitro* and *in vivo* hyperglycaemia have been illustrated as part of the work in this thesis, we set about examining whether changes could be detected in the level of annexin 2 in response to *in vivo* hyperglycaemia. Western blots of isolated brain microvasculature, retina and kidney were performed, although only preliminary samples of brain microvasculature and eye could be obtained. Nevertheless, in all three cases an up-regulation of annexin 2 was detected, with significant differences detected in the kidney, indicating that chronic *in vivo* hyperglycaemia causes effects on annexin 2 expression that differ from *in vitro* hyperglycaemia. Some of the effects seen here could be a function of increased surface expression of annexin 2 since it is known that its levels on the surface of the endothelium increase in response to hyperglycaemia[378]. It is unlikely however that the levels of increase seen, particularly in the brain microvasculature, would be accountable

to increased surface expression alone, hence it is more likely that intracellular levels also increase. Speculations into the reasons why annexin 2 levels increase in diabetes, would focus both on the enhancement of plasmin activity in order to maintain normal haemostasis, potentially compensating for annexin 2 that may have been functionally inactivated by the consequences of diabetes[522], and stabilisation of the junctions between cells that can be affected by the progression of diabetes[349,344].

6.7 Conclusions

The aim of work in this thesis was to examine a hypothetical role for annexin 2 in diabetes, and more specifically diabetic retinopathy and diabetic nephropathy. To achieve this we examined annexin 2 using both *in vitro* hyperglycaemia and *in vivo* models of diabetes, the latter achieved using streptozotocin in the annexin 2 knockout mouse. The preface for such an investigation was taken from the previous observations in the literature that annexin 2 is altered by the consequences of hyperglycaemia both via glycation[375] and oxidative stress[383], and shows responses distinct to those exhibited in normal physiology, such as increased translocation to the surface of endothelial cells[378], were it may compensate for damage incurred as a consequence of diabetes. In addition exogenous annexin 2 has been shown to be effective at preventing diabetic nephropathy, through enhancement of plasmin formation[374]; and hence annexin 2 was identified as potentially crucial in preventing the progression of diabetic microvascular disease.

Since pharmacological inhibition of annexin 2 is not possible, we set about examining a role for annexin 2 in diabetic microvascular disease using the annexin 2 knockout mouse. Using this system, changes were detected in various aspects of *in vitro* endothelial cell biology, diabetic retinopathy and diabetic nephropathy, as discussed above. Three major differences emerged: changes in glucose homeostasis, VE-cadherin localisation and re-absorption of solutes and water, which collectively serve to highlight a common element in this thesis, namely the role of annexin 2 in the targeting and trafficking of proteins in response to stimulus. In all three data sets, loss of annexin 2 resulted in reduced or exaggerated responses of the key proteins involved with the trafficking and targeting roles of annexin 2 as likely causes of the effects seen. Interestingly, subtle differences were additionally detected in AnxA2$^{-/-}$ non diabetic animals, both in the context of glucose homeostasis and albumin excretion. This serves to illustrate another key element of annexin 2 function, that although the annexin 2

knockout mouse is viable with few differences detected between it and its wild-type counterpart, differences may only become detectable in situations in which the body is stressed. This could reflect redundancy within the annexin family, a concept that has been noted in the literature for other annexins[523,524,525], but also shows that at least some of the functions of annexin 2 cannot be fulfilled by other proteins.

These studies have revealed potential roles of annexin 2 in diabetic microvascular disease both in the eye and kidney, and therefore have created areas of annexin research that have not yet explored. The work in this thesis is the first reported use of streptozotocin to induce diabetes in any annexin knockout mouse, and the first to reveal physiological differences in glucose homeostasis in the AnxA2$^{-/-}$ mice in both the presence and absence of diabetes. Further work into the role of annexin 2 in diabetes should focus on the roles of protein targeting and trafficking, utilising the kidney epithelium as a useful model, both in the context of diabetes and normal physiology. In addition, the involvement of annexin 2 in glucose homeostasis opens an exciting new are of annexin research, and studies designed to examine this process would provide new insight into the role of annexin 2 in an area of outstanding biomedical importance.

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